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PHYSIOLOGICAL REVIEWS

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CONTENTS

	PAGE
WOUND HEALING. *Leslie B. Arey*	327
CHRONAXIE. *Hallowell Davis and Alexander Forbes*	407
THE RÔLE OF VITAMIN C IN THE ORGANISM AS SUGGESTED BY ITS CYSTOLOGY. *Geoffrey Bourne*	442
ELECTROLYTES IN MUSCLE. *Wallace O. Fenn*	450
ENDOCRINE CONTROL OF THE MAMMARY GLAND. *Warren O. Nelson*	488

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PHYSIOLOGICAL REVIEWS

VOL. 16 JULY, 1936 No. 3

WOUND HEALING

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The capacity to repair injuries is relatively small in the higher vertebrates in comparison to the regeneration of whole limbs and organs in lower animals. Yet the healing ability is of a high order in most of the tissues, muscle being most deficient in this respect. Normal, physiological death and replacement is an especial characteristic of some tissues—notably epithelium and some representatives of the supporting group. Qualitatively this differs not essentially from regeneration of a pathological type, although the latter does show quantitative and time elements that are of a different order.

The general features of wound healing have been investigated rather thoroughly. The recognition of several types of regeneration in epithelial-covered surfaces and the main principles involved in the reparative process are elementary matters which are summarized accurately in several textbooks of pathology (*e.g.*, Aschoff, 1928). Modern knowledge of these principles rests largely on the comprehensive investigations of Loeb (1898), Marchand (1901), and Werner (1902). The earlier literature is reviewed adequately by these workers, and especially by Marchand. Some of the more recent phases have been discussed by Loeb (1920a), v. Gaza (1926), Häbler (1928), Carrel (1930), Howes and Harvey (1930), and Tammann (1933).

It is customary to differentiate wound healing in the skin into primary and secondary types.

1. Primary Repair. In this type the wound is cleft-like. It may be healed: *a*, through simple epithelial regeneration, if no other tissues be involved; *b*, by direct union of the parts which readily stick together again, suffer no appreciable tissue loss, and permit uncomplicated regeneration of epithelium and of connective tissue (cicatrization); *c*, healing under a scab (consisting of exuded fluid, tissue fragments, etc.) which provisionally closes the wound; this is referred to as "pri-

mary scab healing." These types, and especially the second variety, are also termed "healing by primary intention."

2. Secondary Repair. In open wounds, involving gross defects, the cicatrization is preceded by the building up of "granulation tissue," each granulation representing a minute vascular territory consisting of newly formed, vertically upstanding blood vessels which are surrounded by young connective tissue and wandering cells of different kinds. Such repair of the dermal elements, accompanied by concurrent epithelization, can also take place under a scab (secondary scab healing). Formerly the name "healing by secondary intention" was given to this second group, especially when suppurative phenomena accompanied the granulation process.

It seems unnecessary to recount further these general phenomena which are so well covered in text-book accounts. Instead, an effort will be made to review and organize the intensive studies of relatively recent years which have extended so greatly our information in a detailed and quantitative manner. Naturally enough such advances have resulted largely from the utilization of modern experimental methods of approach. Most of the investigations, both old and new, treat of skin wounds, and unless specific mention is made to the contrary the statements in this review will refer specifically to this type. However, internal wounds of the serous and mucous surfaces differ in no fundamental way from those of the external surface. The more specialized problems of repair in tissue other than those which include the partnership of epithelium and fibrous tissue, such as is found in skin, serosae and mucosae, cannot be entered into in this review.

I. CONDITIONS INFLUENCING WOUND HEALING. *1. External Conditions.* Wounds may be caused by a variety of agencies—mechanical, thermal, chemical and electrical. These, however, for the present purpose do not need individual consideration since there is little fundamental difference either in the injuries inflicted or in the reparative measures which follow.[1] Other variant attendant conditions, which have great clinical importance, are, for the most part, not matters of special fundamental concern. Such include: open and closed wounds; operative and accidental; shape and size; character of the filling material; degree of traumatization and necrosis; septic and aseptic. These external conditions are all largely dependent on the nature of the wounding agent. Over them the physician has some measure of control, and through

[1] An exception is the peculiar type of wound resulting from the high frequency "electric knife" (*cf.* p. 355).

treatment can provide favorable conditions for the proper action of the internal factors. External stimuli having to do with the course of regeneration include such factors as infection, foreign bodies, air, light, temperature, humidity, mechanical and chemical influences.

2. Internal Conditions. Another set of factors has to do largely with the functional activities of the living tissues themselves. This group includes certain fairly tangible factors such as hyperemia (and the conditions of temperature, moisture, acidity, etc., attendant on it), hormone and trephone action, trophic nervous activities, cytotropism, etc. But particularly concerned are the innate processes of regeneration; over such factors there is but little opportunity for control or influence.

Bier (1917) prefers not to group the factors influencing wound healing as external and internal stimulating agents, but rather as "körper- und gewebseigene Reize" and "körper- und gewebsfremde Reize." The same agency may even be "körpereigene" (*e.g.*, gastric juice) but "gewebsfremde" (*e.g.*, gastric juice on deep wounds of stomach).

II. EVENTS OF WOUND HEALING. *1. Provisional Closure. a. The fresh wound.* The primary results of a wounding agent are the dislocation (through cutting and tearing) of previously continuous tissues, which results in a wound of some sort. This is apt to gape because of the retraction of elastic or muscular constituents. In all deep wounds, blood vessels are destroyed and a variable amount of hemorrhage follows; nutritive disturbances of a more subtle nature are then set up in the regions deprived of their normal blood supply. Similarly, many cells in the path of the wounding agent are killed outright, while neighboring ones even to some distance away are more or less seriously traumatized; many of these succumb to necrosis while others recover.

b. Spontaneous cessation of blood flow. As early as 1897 Bier called attention to the activities of severed blood vessels which of themselves bring about the cessation of bleeding. Since then a rather large literature on this subject has accumulated, much of which Stich (1930) has reviewed. The World War presented unusual opportunities to observe and confirm the older testimony that the bleeding of even large arteries can cease spontaneously. Küttner and Baruch (1920) and others have described a segmental spasm in traumatized, large arteries through which the injured free-end becomes contracted and pulseless. Magnus (1923) records amputations at which the ends of large arteries contracted to a lumenless stump and did not leak blood when the ligature was removed from the end. Observations have likewise been

made on laborers, with limbs torn off, in whom the remaining arterial stumps were maximally contracted and free of thrombi; even in the absence of ligatures a significant hemorrhage was lacking. Still other observers of shot wounds through the large arteries record that while the adventitia may show only a tiny aperture, the intima becomes torn away widely and rolls up, rosette-fashion. Contraction and retraction of the vessel wall, rolling-up and entanglement of the intima, and the segmental spasms already mentioned, coöperate as a spontaneous defense against bleeding—and this entirely in the absence of thrombus formation.

Another aspect of the spontaneous checking of blood flow has resulted from the observations of Bier (1924) at operations made bloodless through the use of Esmarch's bandage. The emptied vessel, he maintains, is thereby stimulated into contraction, while the lessened bleeding after release is also due to a blood hunger of the tissues through which blood is drawn away from the artery. Stegmann (1924a, b) has a somewhat similar concept. This investigator has studied hemorrhage under the microscope, using the pancreas and mesentery of living rabbits, and holds that clotting plays only a subordinate rôle in the spontaneous cessation of hemorrhage. He interprets absence of bleeding as being chiefly due to the diversion of the blood stream around and away from the site of injury. This phenomenon Stegmann names the "autonomous reversal of the circulation." Nevertheless, Tannenberg and Herrman (1927), as the result of cutting experiments on small arteries, identify the chief phenomena of spontaneous blood stoppage with the retraction and contraction of the vessel wall itself, and, in the case of the mesentery, on the pressure of perivascular hematomata.

With respect to the smallest vessels, the capillary microscope has made possible direct observations upon the living (Magnus, 1924a). A severed capillary can be seen to evacuate its contents, contract, retract, and remain vanished from sight; thrombosis is never observed. Such behavior has even been recorded by motion pictures made through the microscope (Magnus, 1924b). Hence capillary hemorrhage appears to be safeguarded by the activity of the capillary wall itself, and clotting is not a factor at all. Heimberger (1926) found that blunt pressure used as a stimulating agent causes the contraction of human skin capillaries; their closure may last a day long. He believes the reaction to be due to pericytes.

c. Traumatic inflammation. The events which follow a wounding injury and precede the repair of connective tissue are largely a special

manifestation of the general process of inflammation. In fact, the course of events may be designated traumatic inflammation. This may be either limited in degree or extensive, depending upon the nature and cleanness of the wound. Nevertheless, some inflammation always is present. It is characterized by hyperemia, exudation, leucocytic emigration, etc. These phenomena lead first to the provisional covering of the wound surface with fibrin, and later to the removal of noxious substances, to the resorption of blood and other exudates, and to the removal of degenerating tissue fragments through phagocytosis and enzymic digestion. This second group of activities extends over into the important stages of degeneration and reparation.

d. Closure by apposition. Although clotting may play only a subordinate rôle among the factors having to do with the spontaneous cessation of bleeding, it is, nevertheless, of prime importance in the process of wound healing inasmuch as it provides a provisional closing of the wound. In cleft-like wounds, such as occur in approximated operative wounds, the amount of blood clot is limited but there is a sufficient fibrin sheet to close the wound, stick the opposed surfaces together, and offer an excellent substrate for the invading new cells.

e. Closure by scab formation. Open wounds remain as such only a short while, during which period blood and lymph stream out unhindered. Even within a few minutes a fibrinous mass may be created through the coagulative ability of the blood. At first this is a mere filter which restrains the more corpuscular and viscous constituents of the blood. When this jelly-like exudate, together with entrapped blood cells and tissue fragments, contracts and dries on the surface of a wound, an impermeable crust or scab is formed which thereafter seals the open tissue spaces beneath, and so offers protection against the drying and injury of these tissues until the reparative activities are finished. The process is a typical example of syneresis, like the drying of collodion which thereby loses its capacity to act as an ultrafilter to colloidal substances.

2. Destructive Changes. These activities cover a period which, in the absence of complications, is usually short. It includes chiefly the death and removal of injured cells, and the absorption of exudates.

a. Zones of injury. Borst (1920) distinguishes three zones relative to tissue injury in the wound area. The first zone includes the region in which tissue is destroyed directly by the wounding agent. The second zone is that next remote layer whose tissues are subject to traumatic necrosis. The extent of this territory naturally depends

directly on the type of trauma inflicted; in a cleanly made cut, as by a scalpel, it is of limited extent. The third zone is seen plainest in severe tearing wounds, such, for example, as are inflicted by shell fragments in war injuries. In it the tissues are subjected to molecular violence and the constituent cells experience injury of a sort that may lead to a necrobiosis. Nevertheless, many of the cells of this region are able to recover and participate in regeneration. The last-named zone merges into sound, undisturbed tissue. The first and second zones, especially, become contaminated by micro-organisms introduced by the wounding agent. Of importance in the subsequent process of repair is the fact that the first zone is not surrounded by tissue of full vigor; especially does it make easier the advance of bacteria through such tissues with lowered defense.

b. Tissue destruction. Cells are destroyed in the wound area both directly from traumatization, and more particularly in an indirect manner through metabolic deficiencies which result from injuries to blood vessels and lymphatics. This secondary destruction is accomplished chiefly by autolysis and by heterolysis. These topics are treated in detail by v. Gaza (1918a).

Autolysis acts through enzymic activities which the declining cells themselves elaborate. Among the enzymes are trypsin types and pepsin types (pepsinase; peptase; arginase; etc.). The dissolution proceeds with nearly the same intensity as in autolysis *in vitro* and takes the same morphological course (Brandi, 1926; v. Gaza and Seulberger, 1926).

v. Gaza (1918a) has designated isolysis as an activity similar to autolysis but differing to the extent that the enzymic influence emanates from sound cells of tissue of the same kind.

Heterolysis is especially concerned with leucocytic enzymes and is seen principally in the necrotic zone. Hence heterolytic activity is most vigorous when the necrotic tissue is most abundant; for example, infected wounds show the greatest heterolysis while aseptic wounds have relatively little. Neutrophiles are the enzyme-bearing agents (Opie, 1922; Willstätter, *et al.*, 1930, 1931; Sterne, 1932). Almost nothing is known about similar activities on the part of other blood or tissue cells (v. Gaza, 1926). The active principle of heterolysis, leucotryptase, resembles trypsin and digests under conditions of alkalinity; it cannot attack living cells or unchanged connective-tissue fibrils. In addition to the removal of cells, the fibrinous deposits (which serve as a provisional protective wall both on the surface and in the deeper tissue

spaces) also vanish through the fibrolytic activity of neutrophiles, and presumably of young fibroblasts and angioblasts as well. With respect to the removal of fibrin, as commonly described, should be compared Baitsell's (1915, 1916, 1917, *et. seq.*) view that the fibrinous filaments are transformed directly into collagenous fibers.

Clinically a fourth type of tissue destruction, allolysis, is sometimes important. This dissolution is effected through chemical substances foreign to the normal body, such as lye or bacterial enzymes.

Macrophages (monocytes; polyblasts), by phagocytosing tissue debris and fibrin, play a definite part in the destructive phase of wound healing. Possibly the neutrophiles are active also in removing the smaller particles.

c. Reparative stimuli. The diminished and altered metabolism attendant on tissue destruction is held to play a stimulatory rôle in the reparative process, besides inflicting some injury on otherwise sound cells and inducing an inflammatory hyperemia (v. Gaza, 1922a). The inadequacy of the supply of oxygen and other nutritive substances necessary to life in the ischemic border zone leads to a tissue hunger. The latter furnishes a stimulus (v. Gaza, 1918a) which is known to be correlated with the induction of cell division (Kornfeld, 1922). This stimulatory reaction is common both to vascular endothelium and to the connective-tissue cells, some of the latter responding also by phagocytic activity.

In addition, the products of cell destruction have a stimulative effect. Rix and Greifenstein (1930) record that cultures of granulation tissue from the neighborhood of foreign bodies show a vigorous growth; in these locations there are injured cells. Also Akamatsu (1923) found that cultures of the rabbit's liver grow best in plasma from individuals possessing hepatic wounds. Naswitis (1922, 1924) has provided some preliminary information as to the existence of wound hormones, while Lorin-Epstein (1927) and Fränkel (1928–1932) have studied "necrohormones" emanating from the injured tissues and considered significant in the healing of fractures. With all such influences arising from the products of tissue destruction, should be compared the well-known findings of Carrel (1923, 1930) that definite growth-promoting substances (trephones) are elaborated by leucocytes and are a characteristic of embryonic tissue juices. In practical application, Carnot and Terris (1926), Wallich (1926), Kiaer (1927), Roulet (1927), Schloss (1928), Nakumura (1930) and Sandelin and Björkesten (1932) have proved that injection of such embryonic extract at the wound site does hasten

healing, while Bugliari (1927) and Kiaer (1927) report a corresponding benefit after the application of leucocytes. In contrast to these results should be mentioned the experiments and criticism of A. Fischer (1925). Moreover, Wallich (1926) reports equally good results with Witte's peptone while Kiaer (1927) believes that certain protein decomposition products, the proteoses, applied as a poultice have a similar action. In this they are in agreement with the conclusions of Carrel and Baker (1926). The exact nature of the stimulating agent has been narrowed by Baker (1929) who showed that glutathione and hemoglobin induce fibroblastic proliferation, while Hammett (1929) made a further advance when he identified the sulfhydryl radical as the effective essential stimulus to that kind of growth which operates through an increase in cell numbers. Practical application of this principle has been made in the healing of wounds in lower mammals (Hammett and Reimann, 1929) and man (Reimann, 1930; Birnbaum, 1932).

The fact that the processes of tissue destruction and resorption are frequently paralleled by a period of cellular proliferation is interpreted in a novel way by certain investigators led by Gurwitsch (1926) and by Blacher and his associates. These workers believe that short ultraviolet rays, which have the capacity of stimulating cells into mitotic division, emanate from tissues in which three types of processes are taking place: 1, oxidations (Sorin, 1926); 2, glycolysis (Gurwitsch, 1926; Kisljak-Statkewitsch, 1929); 3, proteolysis (Gurwitsch, 1926; Karpass, 1929). The relation of protein destruction to the "mitogenetic" rays has been studied by Blacher and his co-workers in the metamorphoses of amphibia and insects, in regeneration phenomena, and in wound healing. With respect to the specific application afforded by wound healing the contributions of Bromley (1931), Blacher, *et al.* (1932), and Samarajew (1934) should be consulted for positive findings and the reports of Schreiber (1933) and Helff (1934) for negative results.

d. Wound secretions. Directly following the infliction of a wound the outflowing fluids consist of unmodified blood and lymph. But this condition promptly changes as the fibrin is deposited upon the exposed free surface of the wound, fills the tissue spaces, and to some extent closes the opened lumnia of vessels. Following this temporary phase the fibrinogen content of the early wound secretion decreases until, with the appearance of granulation tissue, it has wholly disappeared. As in ordinary inflammation where the more viscous constituents (fibrinogen; globulin) are prominent, so in the wound secretions of the

first day one finds (in comparison to the tissue lymph and transudate) an increased representation of all the plasma constituents, but especially of fibrinogen and globulin. Within a few days these constituents return to normal and the wound fluid then consists principally of albumin. Always is the wound secretion of an open wound (which is subject to the influence of external factors) distinguished from tissue lymph by a significantly higher protein content and by a more or less considerable admixture of leucocytes. A healthy, granulating wound has a scanty secretion, mucoid in nature and with relatively few leucocytes. The decrease in leucocytes signifies that no more do the destructive changes reign. The physico-chemical characteristics of the aseptic wound secretions have been investigated and reviewed by Lieblein (1902).

e. Rôle of infection and foreign bodies. Every accidental wound becomes more or less contaminated, and even impregnated, with miscellaneous bacteria. If these organisms find conditions favorable for their continued life, growth and spread, then a wound infection is set up. Wounds treated within the first six to eight hours usually can be turned into as favorable a course as those produced by surgical asepsis. In either instance the desired goal is the production of a smooth, germ-free or germ-poor wound surface and the establishment of primary healing (v. Gaza, 1922b). When the infection gains a footing, the destructive stage is prolonged or made to recur, and repair is correspondingly delayed. Thus it is that the destructive phase in wound healing is the dangerous period. The shorter and less complicated it is, the better is the outlook for successful repair. Even the presence of a concurrent infection, such as an abcess, in another region has been noted to exert a retarding influence on a healing wound (Carrel, 1924; Kiaer, 1927).

Other delays besides infective ones are occasioned by large blood clots or foreign bodies. The former hinder the provisional closure of wounds which would otherwise heal by a primary course. The latter, besides being usually bearers of infection, are disturbing elements which if not removed become encapsulated with granulation tissue and later with connective tissue.

3. Constructive Changes. The reparative phase of wound healing naturally overlaps the destructive period. Due to secondary infections or other disturbances the constructive phenomena may suffer relapses and even be forced to give way temporarily to severe destructive recurrences.

A. Connective-Tissue Participation. a. Cells involved. The cells involved in dermal wound healing are those that occur normally in loose connective tissue, together with some derivative types aroused by the inflammatory stimulation. The following summary of the cell types distinguishable in normal loose connective tissue follows the conclusions of Maximow (1927) who probably is our safest guide in a field notable for its confusion and contradictions.

α. Mesenchymal cells. Some fixed, stellate cells of the embryo remain in their undifferentiated embryonic condition. They resemble the fibroblasts, which are more specialized elements derived chiefly from mesenchyme, but are always smaller than the latter. Usually such undifferentiated mesenchymal cells are distributed along the walls of blood vessels; particularly is this true of capillaries where they constitute some of the so-called pericytes. Unlike fibroblasts, which are an end-product, mesenchymal cells are elements with unrealized developmental potencies; under suitable stimulation they may differentiate into new cell types.

β. Amoeboid cells. Some of these migratory elements are identical with the lymphocytes and monocytes of the blood. They may have emigrated from the blood stream or they may be elements which originated in the embryonal mesenchyme and have remained there. Plasma cells (specially differentiated lymphocytes) are rare elements except in certain special locations. All the granular leucocytes are cells of myeloid origin which sometimes leave the blood channels to appear in the loose connective tissue. On the other hand, the mast cells are a specific tissue-basophil which resemble but are not identical with the basophils of the blood. Certain large cells are identical with the smaller macrophages (polyblasts) of inflammation.

γ. Fixed macrophages. These "histiocytes" or reticulo-endothelial cells are normally quiescent elements of mesenchymal origin which possess distinctive morphological features. In addition, they are characterized functionally by their pronounced ability to store electively the particles of acid aniline dyes in colloidal solution.

δ. Fibroblasts. These cells are elongate, flattened elements usually situated alongside bundles of white fibers which are generally believed to have been elaborated under their influence (*cf.* p. 339). They are held to be end-products of differentiation from mesenchyme and are not capable of giving rise to any other cell types, except perhaps to storage fat cells as the result of direct transformation.

In inflammation (including wound healing) certain alterations of

the normal picture occur, so that three cell types then predominate: 1. Neutrophiles, which are emigrants from out the vessels that degenerate promptly after their rôle in the inflammatory process is completed. 2. Polyblasts, so-called because of their diverse appearance and transformations; they are said to arise from lymphocytes, monocytes and fixed macrophages. Among other potentialities they may revert to fixed macrophages again, or transform into fibroblasts. 3. Fibroblasts, which retain their characteristics, divide mitotically, form part of the granulation tissue and later are presumably concerned with the elaboration of the collagenous fibers of the scar tissue. Carrel and Ebeling (1926) have discussed some of their fundamental activities and relations.

In addition, endothelium is an active tissue through its proliferation of new sprouts; this property is especially characteristic of granulation tissue. Endothelium also can contribute to the later fibroblasts of the repair period. On the other hand, its relation to the production of phagocytes and monocytes, as so commonly believed, lacks proof.

The fixed macrophages have varied potentialities. With respect to the inflammatory stimulation they transform into wandering phagocytes (free macrophages) by way of the intermediate polyblast stage, while the amoeboid phagocytes so formed may in turn differentiate into fibroblasts.

Under the stimulus of inflammation many lymphocytes and monocytes migrate out of the blood vessels, increase rapidly in size and transform into polyblasts.

b. Primary healing. The connective-tissue and endothelial cells are proliferating plentifully by the second day. Young cells collect in the wound wall, and after the third day new vascular sprouts are present as well. Extension across the wound space and further filling-in restores the broken continuities between blood vessels and connective tissues. The resolution of fibrin, the formation of connective-tissue fibers and a decrease in the abundance of cells and vessels produce a scar tissue which slowly reduces to a paler, permanent condition only quantitatively different from the surrounding uninjured tissues. At the surface, beneath the new epithelium, typical connective-tissue papillæ are not restored.

c. Secondary healing by granulation tissue. Open, free wounds begin to be characterized in about six to eight days by a "fill" of highly vascular material (granulation tissue) which from the surface has an uneven, pebbly appearance. This is because each wound "granule" has

a vascular tree as a core, growth of which raises the enveloping mantle of wandering cells and young fibroblasts into a hillock-like elevation. The main vessels of the tree are not differentiated into arterial and venous types even though between the afferent and efferent stems there is a network of broad capillaries. Hartwell (1930) has asserted that closely apposed wounds of the human skin, like those of swine, are not commonly characterized by granulation tissue even in the presence of infection. This difference from the condition in leaner animals, such as the dog, guinea pig and rabbit, he believes is due to the presence in man and swine of abundant adipose tissue which encroaches on the wound area and furnishes a substrate for the migrating, reparative cells.

The sheet of young granulation tissue thus provides a cellular protective membrane to the injured body-surface which has hitherto been covered merely by a fibrinous layer produced through the coagulation of the wound secretion. That it is a better protection than the fibrin sheet is proved first of all by its lower permeability (Melchior and Rosenthal, 1920); alcohol, for example, no longer penetrates and elicits painful sensations. Billroth (1865) was the first to proclaim that strong, uninjured granulations protect the body against the entrance of septic substances and infections. Indeed, granulation tissue has been shown to have a definitely bactericidal secretion (Afanassieff, 1897). In short, granulation tissue presents the ideal conditions for a defense against infection; this includes marked hyperemia, active exudation, local leucocytosis, and young, vigorous component cells which lie snugly together and have not yet elaborated paraplastic products.

In contrast to the lack of variability in the healing of aseptic, cleft-like wounds, healing through granulation tissue presents a diverse picture which varies with the conditions under which it originates. Furthermore, the occurrence of infections, necroses, foreign bodies, blood, etc., militates against the elaboration of what is known clinically as "good" granulation tissue. In large wounds the growth force of the regenerating tissue may exhaust itself so that an ulcer develops in the midst of torpid scar tissue. Blood stasis furnishes a fibroplastic stimulus which favors the formation of thick weals.

d. Fibroplasia. The formation of scar tissue is heralded by the appearance of connective-tissue fibrils. In aseptic, apposed wounds this process is recognizable within a few days after the injury occurs. At first the fibrils are sparse; such are apparently richer in water, as they are more delicate than mature fibers, but they soon increase in

number and become firm (v. Gaza, 1918b). In open wounds which are healing by granulation the formation of fibrils depends on the prevailing metabolic conditions. While the destructive phase is still in progress fibrils are not formed. In a similar manner, regions of necroses or areas containing foreign bodies and rich in bacteria have an abundance of cells and are thus not favorable to fiber development. Only when all the dead and foreign material is removed, either by enzymic digestion, phagocytosis or sloughing, does the fibril formation progress. This process proceeds from the deeper levels toward the surface.

Next it may be inquired how union between severed connective-tissue fibers can be effected again. Direct healing by contact and fusion is not possible in such mature structures, since the collagen is so deficient in water and so firm that the fiber bundles cannot weld together directly. Organic union of the borders of a wound is accomplished through the filling in of new connective-tissue fibers which link up with the old. This requires a swelling and solution of the formed substance, both of which changes are strictly dependent on the metabolism of the repair tissue (especially granulation tissue) of the wound (v. Gaza, 1918b). To what extent the collagen experiences only physical changes (swelling; solution) is a matter probably dependent both on the nature of the injury and on certain functional factors. v. Gaza assumes that in the healing of simple, smooth wounds the collagen undergoes no chemical rebuilding but only colloidal changes of state.

The manner of origin of connective-tissue fibers is a long standing controversy, the older phases of which have been excellently reviewed by Merkel (1909). The problem is fundamentally the same whether the new fibers are differentiated in the embryo, in healing wounds or in tissue culture. There are two main schools of thought concerning the origin of fibers and their relation to fibroblasts.

Schwann (1839), Flemming (1891) and others believed that fibrils arose within the cytoplasm itself, and in modern times Meves (1910) has assigned their origin to mitochondria. Nevertheless, this idea of a direct transformation of the cytoplasm proper is no longer held in its original sense. Instead, the theory has been modified by Hansen (1899), Mall (1902), Studnička (1907) and Heidenhain (1907), whose views have most recently been upheld by W. Hueck (1920), Laguesse (1921) and others. These workers assume that the fibers develop in a peripheral, homogeneous ectoplasm which takes the form of a syncytial spongework. In such a syncytium argyrophilic networks are said to appear as the result of vital metabolism on the part of the

syncytium itself. The original ectoplasm is believed to remain as the cement and amorphous ground substance. When the fibers eventually lose connection with the mother cells, the latter continue to exist as mature fibroblasts. The continued growth of such young fibers is supposed to be the result of their intrinsic properties of independent metabolism and growth. Nevertheless, a strong objection to this interpretation is the lack of real proof of the existence of any clear, homogeneous ectoplasm such as is postulated. In living or properly fixed tissue cultures it is not demonstrable (Maximow, 1929). There is suspicion that the effects often produced in fixed and stained preparations are coagulation artefacts.

Contrasted with all intracellular origins is the still earlier idea of a sort of crystallization from a semifluid, amorphous, secreted substance which occupies the intercellular spaces. Henle (1841), Kölliker (1850–1854), Ranvier (1875), v. Ebner (1896), and Merkel (1909) are early names associated with this interpretation. Recently, the list of adherents has been supplemented by Baitsell (1915, 1916, 1917, *et. seq.*), Nageotte (1919), Harrison (1925), Heringa and Lohr (1926), Alfejew (1926) and Maximow (1929). It is safe to state that the intercellular origin has gained ground steadily in recent years. Some, like Baitsell, maintain that the intercellular origin is proved beyond doubt. Others, like Maximow, consider the matter still unsettled, and beyond final settlement until new methods of attack are devised better than the present stalemate techniques.

An intercellular origin is easy to understand. Numerous observations, both old and new, have recorded how the first fibrils in developing connective tissue may appear in a structureless jelly far distant from any cells. The jelly is undoubtedly a lifeless substance, a colloidal sol secreted by the cells themselves. The gelation into fibrils is brought about by some unknown factor, which might be assumed to be an enzyme passed into the originally fluid jelly from the bodies of the fibroblastic cells (*cf.* Harrison, 1925). Studies on wound healing (Baitsell) and on tissue culture (Maximow) confirm these interpretations. Although the delicate networks of argyrophilic fibrils may follow the surfaces of some fibroblasts, they also extend far beyond them and may lie free in the ground substance as well. Growth of the fibrils and consolidation into wavy bundles serve to produce the ordinary gross features of white fibers. The more mature fibers finally fail to impregnate with silver. Baitsell (1915, 1916, 1917, *et seq.*), Nageotte (1916), Harrison (1925), and Hertzler (1929) believe that collagenous fibers

of wounds, embryos and tissue cultures are the direct results of coagulation processes in fluids. All such phenomena are exactly comparable to the clotting of blood plasma. In plasma clots, for example, Baitsell believes he has demonstrated beyond doubt the direct transformation of fibrin filaments into typical collagenous fibers and holds this to be the fundamental feature in fibrous reparation. Maximow (1929), on the other hand, thinks the fibers described by Baitsell do not have all the characteristics of collagenous fibers. Aside from staining reactions, relatively little is known of the chemical nature of fibrin or collagenous fibers, so the chemical identity of the two remains unproved.

c. Scar tissue. The speed with which cicatrization is completed depends on several factors, such as the size and nature of the wound, and freedom from infection and other complications. Surgical wounds of the abdominal wall with good apposition are "healed" within two or three weeks. The process of repair as expressed by fibroplasia has been measured on the basis of the return of tensile strength by Howes, Sooy and Harvey (1929). In wounds of the skin, fascia and stomach the normal strength was found to be regained in 14 days.

However, the young scar is at first rich in cells and blood vessels and only gradually reverts to the permanent type of paler, less vascular and less cellular scar tissue. Although a period of enlargement by spreading follows the close of epithelial healing (Carrel and Hartmann, 1916), a final phase of progressive shrinkage is a well-known characteristic of scars. According to Marchand (1901), the collagenous fibers of old scars, as in mature fibrous tissue in general, differ from young fibers by being doubly refractive; his studies of scars twenty years old showed almost no difference from the normal, surrounding tissue. Nevertheless, the fully healed scar of the skin is often recognizable since it remains a fibrous mass which usually lacks certain characteristic components, like cutaneous glands and hair follicles (Lebram, 1904), and fails to reform typical dermal papillæ. In addition, elastic fibers may be entirely absent according to some authorities (Tammann, 1933), but this is not usually the case. The time when elastic fibers reappear in the scar tissue is variable. v. Schröter (1902) found instances of a beginning restoration at 36 days and a plentiful number in three to six months; in other scars their appearance was delayed for nine months and in still others there were no elastic fibers present after a year's time. As holds for the building-up of scar tissue in general, the mechanical conditions are of great weight in the development of new elastic fibers, while the presence of foreign bodies can retard their growth

(Marchand, 1901). The first fibers appear at the periphery as fine elements which later enlarge and extend centrally. New fibers surround the cut ends of old fibers but do not fuse with them (Passarge, 1894), although Jores (1907) intimates that union does occur. According to the former investigator the appearance and arrangement of elastic fibers in old scars (10 to 30 years) differs in no way from those in the neighboring skin.

The pigment apparatus returns into the scar only incompletely or not at all. In lower animals (frog, guinea pig) the pigment regenerates first in the epithelium and later in the cutis layer (Loeb, 1911). Nerves regenerate readily, and under favorable conditions of wound healing reach the epidermis by the end of the third week (Nasaroff, 1925). The more complex types of nerve ending (Pacinian and Meissner's corpuscles) were not observed to reform either by this investigator or by Leriche and Howes (1931) who also give a general account of the scar and its progressive changes with time.

B. Epithelial Participation. a. Method of repair. In minor, superficial wounds involving the epithelium alone, such as result from excoriations, this tissue is solely concerned in the repair process; new cells proliferate and then are transformed into the definitive types much as in ordinary growth and replacement. Essentially the same course follows vesiculation of the epidermis as the result of burning or blistering agents. In this instance the impermeable horny layer elevates by the accumulation of serous exudate and serves as a protective cover under which the deeper, moist layers proliferate rapidly and restore the original stratification.

Only slightly more complicated is the repair of those cleanly made wounds in which the apposed sides stick together again. Healing is accomplished by reunion of the edges and resumption of the proper continuity of corresponding strata. The restoration of an otherwise intact epithelium may be completed by the end of the first day. Less direct is the resumption of the normal condition in gaping wounds made by cutting or in stitched wounds with faulty approximation of the edges. Here cell migration is necessary, and this is begun promptly; the earliest indications are evident within the first hour. Soon tongue-like processes may be seen which progress over the fibrinous substrate filling the gap until the hiatus is again bridged. The time required naturally depends on the size of the defect and on the kind of obstacles encountered. In tiny wounds of the epidermis of fishes and amphibia epithelial continuity may be regained in as short a period as three hours

(Arey, 1921, 1932a) while corneal lesions of mammals may be healed in six hours (Arey and Covode, 1937). On the other hand, extensive defects and granulating, ulcerous and infected wounds may prove refractory and require months or years before healing is complete.

Much more complex are the conditions introduced by such gross injuries as require the formation of a scab, whether this repair belongs to the primary or secondary method of healing, and the replacement of considerable epithelium beneath it. Equally complex are those secondary repairs over granulation tissue in the absence of a scab. Most of the descriptions which follow will refer to the severer sorts of epithelial healing in which rather extensive regeneration is necessary.

Although contraction of the wound and some other factors may play a part in the process (*vide infra*), at least final restoration of such epithelial defects is brought about by the movement of epithelial tissue from the intact border zone over the connective-tissue or other substrate. Advancing from all sides these modified, reparative, epithelial sheets meet and then return to their normal state of organization. Many conditions exist which serve to vary the details of this process and the time in which it is completed. The size and shape of the wound; the preparation of a proper bed in secondary repair; the occurrence of mechanical obstacles; the presence of infection; the kind of epithelium involved (simple or stratified)—all these, and others, are definite modifying factors, so that a single description will not apply comprehensively to every condition encountered.

The most intensive study of the morphological aspects of regenerating epithelium has been furnished by Loeb (1898) whose account, describing experimental wound healing on the ear of the guinea pig, has been confirmed in many essential details by Werner (1902). From this report much of the following account has been drawn, especially as regards healing under a scab.

The extension of epithelium from all sides is in progress even on the first day. The granular and horny layers of the nearby epidermis disappear as such and resolve into a homogeneous, syncytial layer with dark nuclei of a characteristic rod-like form. This covering sheet Loeb has designated the "upper protoplasmic layer." It migrates the fastest of all the epithelial components and rapidly makes a covering to the whole scab; even at 36 hours this response is well along toward completion. Since the upper surface of the scab is not smooth, one finds that the rod-like nuclei both aggregate above it and fill its indentations. The exposed surface of the protoplasmic layer, on the other

hand, is a glassy, protoplasmic membrane, almost devoid of nuclei. The whole syncytial covering is lost with the scab when the latter falls off. The same protoplasmic layer also invades the scab from the sides and from above downward, dividing into arms which take diverse directions. The syncytium appears gradually to dissolve in an enzymic manner the scab substance, including islands of blood, encountered on the way. This epithelial tissue clings firmly to the scab which it has penetrated, so that if the scab be torn apart separation occurs through splitting of the protoplasmic tissue rather than of its own.

The cells of the Malpighian layer (including the basal, columnar cells)[2] in their wandering assume an elongate, spindle shape, with correspondingly stretched, vesicular nuclei. The long axis of a cell naturally coincides with the direction of its movement. Fibrils appear within the cells, oriented with the main axis. Other characteristics are a marked volume increase of both cell body and nucleus, while cell boundaries become indistinguishable. These elements not only invade the scab from the sides but also pass beneath, between it and the underlying connective tissue. Advance cells are sometimes arranged in tandem series, apparently making their way by means of a local solvent action. Behind them follows a compact mass of epithelium, mostly in the form of an advancing wedge. The upper side of the wedge tends to be covered by the syncytial upper protoplasmic layer, already described. The upper Malpighian cells usually progress faster than the deepest ones and so come to constitute the apex of the wedge. Within the scab diverse appearances are seen, depending on local conditions. Branching epithelial arms take different directions. Clotted blood is enclosed and dissolved, the spaces so created then being organized by the epithelial tissue; similar epithelial organization takes place beneath the scab in the connective tissue. In the first two weeks the epithelial tongues adhere more firmly to the scab than to the connective tissue, so that they cling to the scab in the event of the latter being lifted up. This compact epithelial mass which has not only invaded the scab and passed beneath it, but also has resorbed and organized it, is retained when the scab falls off; thereafter it constitutes the new, definitive epithelial covering.

Marchand (1901) has described the general appearance of epithelial ingrowth over a granulating wound, such as may be discerned with the

[2] Werner (1902) does not believe the basal cells participate in this migration, except as the result of a late, passive slipping due to pressure exerted by horizontal mitoses.

unaided eye or hand lens. The delicate epidermal border is pale blue, terminating as a smooth though somewhat wavy line. Little irregularities on the surface are caused by the granulation tubercles beneath. In older specimens the epithelial border is commonly demarcated from the growing granulations by a seemingly deep groove. The border adheres rather firmly to the surface, so that when raised a thin layer of granulation tissue remains attached. A short distance farther back the firmness of this union decreases. In vertical sections the epithelial border is often considerably thicker than the surface view might have suggested. For the most part the edge is somewhat thickened, while it may continue beyond the border proper as a still thinner flange.

According to Loeb's interpretation a large part of the transparent, nuclear-free masses in the scab consists of the altered upper protoplasmic layer together with portions of the original scab substance which have been transformed by the resolving activity of the invading epithelium, as already described. Such an origin of the latter tissue may be further indicated by the presence of nuclear traces. This interpretation is in opposition to the earlier view which had assumed an origin from clotted transudate emanating from the tissue beneath. Loeb also contends that a great part of the nuclei of the scab, which others have held to belong to leucocytes, is actually of epithelial origin. They are recognizable as such by their rod-shape and, in pigmented skin, by the retention of pigment about them. Due to the unfavorable conditions for the maintenance of life which exist in the scab, such nuclei gradually succumb and disintegrate into granules.

The branching and irregular manner of growth of regenerating epithelium within the scab has been mentioned. In addition, cysts and other atypical growths may occur. To what extent epithelial processes can extend downward into the underlying connective tissue is doubtful (Loeb, 1898), although atypical growths can be found in such locations. It is possible to explain some of these on the basis of local connective-tissue invasions and organizations of the scab (Cohnheim). Moreover, it may be that the connective tissue just beneath the scab is injured, or at least subnormal, through its temporary freely exposed condition or as the result of other influences. However this may be, Loeb believes that the downgrowth of atypical epithelial processes in these locations is a reality and that connective tissue so encountered may be dissolved, as was the substance of the scab. Furthermore, similar epithelial growths can invade growing connective tissue such as is active in filling in a defect. It is interesting to note that even cartilage when encountered can be invaded and destroyed.

At first there is no union between regenerating epithelium and the connective tissue, or, for that matter, with the scab; but such a connection has to be created. It is not easy to decide how this union between young epithelium and delicate granulation tissue is accomplished. Loeb (1898) describes how, after epithelial intrusion into connective tissue, the fibrillae of the epithelial cells brush out, divide into finer fibrils and pass without interruption into what may be called connective tissue. But this investigator further holds that fibroid networks exist beneath the epithelium, which not only are continuous with the epithelium but are actually transformed from it. Even part of the nuclei of this territory are interpreted as of epithelial origin. Marchand (1901) likewise stresses the fibrillar content of the lower epithelial layer and the way the cell bases cling to a delicate basement membrane; the latter is of the nature of connective tissue and connects directly with the fibrillae of the granulation tissue. However, he is not convinced of the existence of a union between the protoplasmic fibers and those of connective tissue. Werner (1902) merely notes the appearance of fibrils which effect a union of the two tissues, but gives no details.

The union of the epithelial plates growing in from opposite sides in regeneration may be regular and even, so that corresponding levels and layers join again. But this is not always true, and dissimilar layers may come to unite as the result of an irregular meeting. In this instance heterogeneous cellular elements are at first in line, but soon this confusion is remedied and the order of layering made consistent. The method by which this return to homogeneity is accomplished is important. It appears that dislocated cells do not move about to reassume their former positions. On the contrary, there is no cell specificity according to original layers of origin. The only specificity is that the upper protoplasmic layer can neither be supplied by the Malpighian layer nor be transformed into it (Loeb, 1898). Cells of the Malpighian layer coming to rest in positions other than they formerly occupied simply assume the characteristics of those levels.

The growth tendency of connective tissue is restrained by the covering epithelium. It is doubtful whether the upper protoplasmic layer has this influence, since it grows in very quickly while the proliferation of connective tissue is still strong. Apparently it is the entrance of the Malpighian cells that ends the invasive proliferation of connective tissue (Loeb, 1898). The operative force in this antagonism is not known.

b. Mechanism of closure. α. Rôle of migration. The chief biological factor responsible for the extension of epithelium over the denuded area is the amoeboid movement of the neighboring cells themselves. This, however, is not the only explanation of wound closure that has been advanced. Some have assigned the cause wholly to cell proliferation (Eberth, 1891; Bardeleben, 1901), while others recognize both mitosis and increase in cell size as minor contributory factors (Werner, 1902; Loeb, 1920a). Still others have invoked certain mechanical forces which force the epithelium centripetally in a passive manner (Garten, 1895; Born, 1897); better established is contraction, due either to shrinkage of the young granulation tissue (Carrel, 1910) or of the young cicatrix (Loeb, 1920a), to shrinkage of the wound margin, or to unknown forces within the old marginal epithelium (Burrows, 1924).

Klebs (1875) and Peters (1885) were the first to advocate the rôle of amoeboid cellular activity. At present various sources of confirmatory evidence are available (Arey, 1932a; Herrick, 1932). First of all, the growth of epithelium in tissue culture affords some fundamental evidence. Sheets of epithelium are formed if an appropriate substrate for this growth be furnished. In the earlier stages of the process cell proliferation is not found, but it does occur later (Oppel, 1912; Holmes, 1914).

The process of ectodermal wound healing has been followed in the chick embryo by Poynter (1919) who finds that a dedifferentiation to an indifferent cell type occurs at the wound margin. A general movement of these cells then brings about healing. Only at a later period is cell multiplication considered to be a factor in wound repair. Furthermore, Holmes (1914), working with larval and adult amphibia, records the direct observation of moving pseudopodial extensions of the living epithelium, and these he regards as adequate to accomplish all the epithelial shifting.

The repair of wounds of the corneal epithelium is accomplished with great rapidity and shows all indications of being due to an active creeping of the cells involved (Arey and Covode, 1937). In fact, these investigators found an actual decline in mitotic frequency during the first days following an injury.

Among the early opinions on skin lesions should be mentioned the view of Born (1896), who recognized that the period of epithelization in amphibian larvae was insufficient to be accounted for by mitosis, yet thought the cells were shifted by individual cell flattening rather

than by active wandering. Even more convincing evidence of the inadequacy of mitosis is furnished by certain natural experiments in fishes and amphibia (Arey, 1932a). Although several thousand cells may be involved in the formation of cysts built around tiny, biting molluscan parasites, the frequency of mitosis is negligible. It cannot be even a contributory factor of any significance during the period of about three hours in which repair is effected. In these instances the migratory movement is somewhat of a mass nature, even though fundamentally amoeboid, for goblet cells and pigment cells are swept along with the less specialized types. Not only do epithelial cells advance into the encapsulating cyst, but the excess marshalled in an over-reaction apparently return to the main tissue in the same manner (Arey, 1932b, c). Large wounds in the skin of fishes also heal rapidly (12–36 hours) in the absence of mitosis either in the epidermis or connective tissue (Harabath, 1928).

Loeb (1898), Marchand (1901), and Werner (1902) likewise observed the movement of epithelium over mammalian wound surfaces and concluded that this must be the result of an active wandering since mitoses were not increased during the early stages and especially did they not occur in the cells of the border zone. Although regeneration of some internal epithelia (*e.g.*, the gut) is not so vigorous as that of the skin, a rapid reaction is exhibited by the postmenstrual repair of the human endometrium. Novak and Te Linde (1924), observing especially favorable material, record the absence of mitoses in this tissue during the period of rapid restoration.

In view of all the available information, the primacy of cell migration in the epithelization of wounds cannot be doubted, even though the contraction advocated by Carrel (1910) and Burrows (1924) may lessen the diameter of the original lesion markedly in some locations.

β. Rôle of proliferation. Modern investigators agree in failing to find a significant increase in cell division during the earlier stages of epithelial repair when it might theoretically explain the prompt, initial ingrowth over the denuded area. Proponents of the view that such an early proliferation is responsible for epithelial movements include: Fraisse (1885), whose own material did not offer any supporting evidence; Eberth (1891), who assigned the proliferation to the free, growing border; and Bardeleben (1901), who believed that border proliferation is reinforced by mitoses in regions farther away. On the other hand, the evidence against this view as an adequate explanation of epithelial movement and wound coverage is overwhelming (*cf.* Arey, 1932a).

Those who have found mitotic cell proliferation significantly increased only at a later period rather than at the beginning of regeneration (and often some distance away from the growth zone) include the following: Lazarenko (1924), Zawarzin (1927), and Danini (1928) on the epithelization of celloidin foreign bodies introduced into invertebrates; Oppel (1912) and Holmes (1914) on epithelium in tissue cultures; Poynter (1919) on wounds of embryos; Oppel (1913) and Osowski (1914) on explants of embryos of the trout, chick and tadpole; Gurwitsch (1926) and Arey and Covode (1937) on the cornea; Arey (1932a) on gill- and skin-wounds of fishes and salamanders; Loeb (1898), Marchand (1901) and Werner (1902) on mammalian skin; Hartwell (1929) on human skin; and Novak and TeLinde (1924) on human endometrium.

Some quantitative data are available. In the wound healing of the stratified gill epithelium of Necturus, Arey (1932a) found the mitotic activity to be increased 135 times during the week following the completion of wound healing which of itself consumed only about 12 hours. Moreover, Arey and Covode (1937) have followed the proliferative activity in corneal wounds and have calculated in serial sections the number of mitoses per cubic millimeter occurring at different stages. During the period of epithelial repair the frequency falls off to less than one-half the normal amount. Although wound closure was complete in six to ten hours it was not until after the fourth day that the rate returned to normal. This actual decline following the infliction of a wound, and perhaps of the nature of a shock phenomenon, is a phase of the process which seems to have been overlooked hitherto. However, during the fifth day there was a marked acceleration, to exceed the normal rate by 75 per cent, followed by a return to normal.

In larger wounds, with a longer healing period and more new tissue to be supplied, the mitotic history is somewhat different (Spain and Loeb, 1916; Loeb, 1920a). During the early stages of repair the mitoses increase markedly in the old epithelium (especially near the border) as long as the wound is open, and the number is usually proportionate to the quantity of cells which have moved into the defect. In the outgrowing epithelium, on the other hand, there are few mitoses at first, but a sharp rise appears after the first few days and reaches a maximum at the time of closure.

The assigning of amitosis as a factor in regeneration belongs to the period when this method of cell division was accepted with a less critical attitude than today (Loeb, 1898; Marchand, 1901; Werner, 1902). That double nucleoli and nuclei, as well as stages of nuclear constric-

tion, have no relation to the alleged division of cells by amitosis has been shown in living cells by Macklin (1916). Conklin (1917) has presented much additional evidence, and after a thorough analysis of his own data and those of others concludes: "There is not a single conclusive case in which amitosis has been shown to occur in the division of normally differentiating cells." In wound healing on Necturus no evidence of amitosis could be found by the present writer (Arey, 1932a), although the large cells of this animal offered especially favorable material for its detection. It would appear, therefore, that amitosis may be dismissed as a significant factor;[3] seemingly illustrative stages can as well be interpreted otherwise or perhaps relegated in part to necrobiotic alterations.

γ. *Rôle of volume increase.* During epithelial repair the cells and their nuclei gain in size both in the old epithelium adjoining the wound and in the new covering layer (Spain and Loeb, 1916; Akaiwa, 1919). The average gain is about 35 per cent, and this maximum is attained just prior to the closure of the wound. The size increase contributes in a minor way to the forward extension of the growing membrane, besides adding to its thickness (Loeb, 1920a).

δ. *Rôle of contraction.* In addition to the intrinsic, vital factors already summarized there is another whose importance, in the skin at least, seems to vary with the looseness of attachment to deeper structures. In the ear, as an example of locations where the skin is fixed to deeper, rigid structures, contraction is a late phenomenon characteristic of the period of wound closure and afterward (Loeb, 1898). These results have been interpreted on the basis of a secondary contraction of the connective tissue which proliferates early in the floor of the wound (Burrows, 1924); hence such contraction is of the nature of a final act and results from a regression of the cicatrix. Somewhat similar is the history of infected, granulating wounds on which the overgrowth of connective tissue is excessive. After the fill of granulation tissue and the removal of the infection, the new epithelial membrane spreads over the wound. The granulation tissue suffers simultaneous shrinkage and draws the edges of the wound closer together.

Carrel (1910, *et seq.*), on the contrary, has arrived at different conclusions from studies on the loose skin of man and various other mammals. He finds that contraction enters after a quiescent period of two to five days and proceeds fastest in the early days that follow; gradual slowing

[3] For a recent renewal of this controversy with respect to the epidermis the reader is referred to Grynfeltt (1931) and Mannes (1934).

then occurs until the contraction ceases when the edges of the epithelium are about 10 to 15 mm. apart. Carrel believes that the process of contraction is the most important factor in the healing of large- and medium-sized wounds and refers to it definitely as a granulous contraction. On the other hand, it is negligible or absent in wounds of 10 mm. or less. Epithelial migration may follow the contraction period as a separate phase, or after a period of contraction the two may progress together (Carrel and Hartmann, 1916).

Somewhat similar are the observations of Burrows (1924) on wounds of the mobile skin of the trunk and extremities of rats and guinea pigs. After six or more hours the edges of the wound begin to move together (but, according to this investigator, with progressively increasing speed) until closure may be practically effected. Both epidermis and corium are involved, and the contraction continues to the limit of their elasticity, or until the skin is fixed by the connective-tissue overgrowth in the intervening gap. Only later does he observe the extension of an epithelial membrane over the free surface of the granulations to complete closure. Thus a large part of the wound is covered by the stretched skin of the margin, without any "gross evidence" of the formation of new tissue. Some additional lesions were made in the firmly anchored skin of the neck and ears. In this instance the migration of epithelial cells was prompt and early shrinkage meager, as Loeb had described. Admitting that such forward movements of the skin as a whole may result from shrinkage of the wound margin, Burrows, nevertheless, believes that it is the result of an intrinsic epithelial force associated with cell proliferation. His evidence for the latter interpretation is somewhat inadequate and unconvincing.

ϵ. *Conclusions.* Of the several factors just discussed, two only are of prime importance in the rapid restoration of epithelial continuity. One, contraction, plays an important part in reducing the area of an open wound greater than 10 mm. in diameter. The maximum effect of contraction is found in mobile skin which is loosely attached to the deeper structures, and in this instance the contraction affects both epidermis and corium as a unit. The cause is presumably identified with some mechanical factor such as shrinkage. In more firmly anchored skin, contraction plays a less important rôle; it then makes a late appearance and is associated with the regression of the cicatricial tissue.

The second factor is cell movement, apparently by amoebism. Mitosis is not a feature of the initial stages and in small wounds may not

show any increase until after the epithelization is complete. In such instances the mitotic region may be quite outside the repair area, and this activity can then be interpreted as compensatory and for the purpose of restoring cells lost to the wound by emigration (Arey, 1932a). If the lesion be small enough and the supply of neighboring cells large enough, the mitotic phase may never become detectable as such. In wounds so large that the adjoining epithelium cannot supply sufficient cells within a comparatively short time, cellular proliferation then enters before epithelization is complete, and cell movement and proliferation go on simultaneously.

c. Quantitative factors in epithelial healing. Loeb and his associates have supplied various details of a comparative nature based on studies of wound healing in the epidermis of the guinea pig, rat and pigeon (Addison and Loeb, 1913; Spain, 1915; Spain and Loeb, 1916; Akaiwa, 1919). The chief comparisons and conclusions have been made more conveniently accessible in a general recapitulation by Loeb (1920a) where the fundamental factors have been analyzed and separated from the trivial and accidental phenomena. To a certain extent some of the data may be peculiar to wounds in epidermis that is firmly attached rather than freely mobile, since Carrel (1910) first found and Burrows (1924) later emphasized certain points of difference in the behavior of the latter type. However, these are the only quantitative data available and, on the whole, are probably applicable to wound repair in general.

α. Cell movements. The rate of epithelial movement depends, among other factors, upon the size of the wound. The epithelial tongues extend faster in smooth and shallow wounds than in uneven wounds or those in which a firm adherence of the scab presents obstacles to the movements of the cells. This epithelial movement is most energetic in the guinea pig and weakest in the pigeon—which corresponds also to the proliferative energy of the normal epidermis of these animals. Nevertheless, there apparently exists in addition a reserve power which makes possible an unexpected manifestation of energy on the part of a normally less active epithelium.

Comparing the rate of growth of the epithelial tongues at different periods it may be provisionally concluded that, while variable factors complicate the result, on the whole a direct proportionality seems to exist between the length of the tongue at any moment and the time period during which it has been growing. This proportional relation is independent of any particular phase of the healing process.

The rapidity of epithelial growth varies directly with the size of the wound according to Spain and Loeb (1916), but with an inverse relation according to Carrel (1910). The apparent discrepancy in these two sets of observations probably depends on the relative importance of contraction in different locations, which in turn is related to the firmness with which the epidermis is anchored to underlying tissues (*cf.* p. 351).

β. Mitotic proliferation. In the three animals under comparison the normal mitotic frequency in relation to the same total number of cells is highest in the guinea pig, less in the pigeon and least in the rat (22: 14: 8). In a unit area, regardless of cell size, the guinea pig again leads but the rat and pigeon interchange places; a similar rating holds during epithelial healing, so that as with cell migration the epidermis of the guinea pig is most active and the pigeon most sluggish.

In all three animals mitoses are rarer during the first several days in the new epithelium than in the normal skin, but in the old epidermis adjoining the wound they increase as early as the second day. The number of mitoses then increases in the new epithelium and reaches a maximum just before wound closure (between the fourth and eleventh day); this in turn is followed by a decided decrease following closure. The temporary mitotic activity extends from the wound margin backward into the old epithelium with gradually decreasing intensity. In the majority of instances the proliferation in the old epithelium is most marked in the earlier rather than the later stages of repair—a reversal of the condition just cited for the new epithelium. The greatest absolute proliferation is in the new tissue, but the maximum per unit area is in the old.

The absolute and relative number of mitoses may be greater in large and deep wounds. This is true for the rat while the evidence from the guinea pig is not entirely satisfactory and needs further clarification. This additional information is especially desirable inasmuch as both cell movements and cell size reach a maximum in large wounds of the type studied by Loeb and his collaborators. To a certain extent there seems to be a direct relation between the number of emigrated cells and the number of mitoses in the old epithelium adjoining the wound. This, however, appeared to be limited to the early stages of healing and to tongues of the same wound. Comparisons between wounds of different individuals and different species do not show the same relationship at all clearly, probably because masked by variable responsiveness and other factors.

That entirely different relations between the time, rate and place of mitosis in comparison to cell migration may exist in small, quickly healing wounds has been established by the present writer elsewhere in this review (p. 349).

γ. *Structural characteristics.* The size of the cell body and its nucleus during wound healing offers some significant information. The variations of these measurements follow closely the variations in the numbers of mitoses, but cell and nuclear size are more constant and dependable factors since less liable to rapid change than the mere number of mitoses.[4] The greater maximum sizes are attained in the larger and deeper wounds, as was the case with mitotic frequency. Also conformable with the results on mitosis, the maximum size is reached just before or at the time of closure of the wound; the cells nearest the center of the wound reach their maximum somewhat later than the cells in the old epithelium, and the greater the increase in cell size near the original wound margin the farther it extends backward into the old epithelium. Comparing the three animals it has been determined that for the normal epidermis the guinea pig has the largest nucleus in comparison to total cell size and the pigeon has the smallest. During wound healing the cell body outgains its nucleus in the guinea pig while the reverse is true in the rat and pigeon. The percentage gain for both cell and nucleus ranges between 30 and 40 per cent.

The stratum germinativum thickens during wound healing not only by an increase in cell size, and in the number of cell rows through mitosis, but also by the emigration of cells from out the old epithelium. Again it happens that, on the whole, the maximum thickness is reached at the time of wound closure. However, at this period the place of greatest thickness is located at the insertions of the epithelial tongues on the old epidermis, whereas the maximum cell size (and probably mitotic activity) is in the old epithelium itself. If, as in the rat, the larger wounds close slower than smaller ones, then the germinative layer becomes so much the thicker, while in the guinea pig there is little difference in the larger and smaller wounds, both of which close rapidly. As has been observed for certain other features, the thickness of the stratum also seems to be greater in deeper rather than shallow

[4] That mitotic activity is a rhythmic phenomenon with periods of relative activity and rest has been shown in both embryonic and adult tissues by various investigators (Sabin, 1920; Kornfeld, 1922; Droogleever Fortuyn-van Leyden, 1924; Picon, 1933; Carleton, 1934). These periods of acceleration and depression offer a possible source of error in all determinations on local regions.

wounds. Comparing now the three animals, one finds that the germinative layer of the normal skin is thickest and has the most cell rows in the guinea pig, while these features are least developed in the pigeon. Despite its initial, intermediate position the rat outgains the other two in both respects during skin regeneration.

Studies on the variations in the number of cells present show that such variations parallel those of cell size, mitotic activity, thickness of the germinative stratum and number of cell rows. In all these details maximum values are attained at about the time of wound closure, and sooner in the old than in the new epithelium. As with cell size, the variations in cell numbers in a unit area during the several periods of wound healing are relatively smaller than is the case for the number of mitoses, thickness of the stratum germinativum and number of cell rows. The absolute number of cells is greatest in the new epithelium but the number to a unit area is largest in the old epithelium adjoining the wound. Here, too, the size of cells, and probably the frequency of mitosis, is maximum.

δ. Closure time. The time of closure of the wound shows marked variations in different species and under different conditions in the same species. The pigeon, in which all the reparative activities are more sluggish, proved to be the slowest to effect closure, while the guinea pig was fastest but not much more so than the rat. Thus, in the experimental wounds, the fourth and fourteenth days marked the limits of closure times. In addition, a certain parallelism could be established between the intensity of cell activities in the normal and the regenerating skin, on the one hand, and the closure time on the other. At fourteen days the epidermis has nearly returned to its normal condition, especially in the fastest closing wounds; cell size, mitoses and thickness of stratum germinativum were then essentially normal in the guinea pig, but somewhat above the ordinary level in the rat and pigeon. The duration of the healing cycle is, therefore, approximately the same in all three animals—slightly more than fourteen days. Within the experimental range used, this period was independent of the size of the wounds.

4. Electrosurgical Wounds. Certain peculiarities are exhibited in wounds produced by the employment of high frequency currents in surgical operations.

a. Stanching of blood. The phenomena incident to the stanching of blood flow have been investigated by Bauer (1931). In large vessels the electrocoagulative effect of Joule's heat produces visible and per-

manent maximal contraction of the stump, the blood column becoming pressed back into the unaffected vessel; at the same time there is marked retraction of the free stump. In smaller vessels and capillaries the coagulative action is highly efficient and bleeding essentially eliminated. It is this that renders the electric knife so valuable in the surgery of especially vascular organs, such as the brain, lung, liver and spleen. Microscopical study of the rabbit's ear showed three zones, separate in time of appearance and in histological structure. Nearest the path of the electric knife there is both contraction and retraction of a blood vessel and closure of its lumen by a coagulation border. Farther off the component tunics of the vessel wall become approximated and fused, layer to layer, in such a manner as to strengthen and supersede the primary closure. A third zone, most remote, is characterized by a thrombus which first serves as a closing plug to the vessel's stump but soon organizes to effect a stronger and more permanent seal. The whole process of blood stanching is, however, endangered by the demarcation of the coagulation border, which is instituted at about the fifth day. The resulting demarcation zone passes midway through the stump; a casting off of all the distally situated necrotic and necrobiotic tissue can lead directly to the postoperative bleeding which various observers have reported as a characteristic of the electrosurgical method (Heymann, 1930; Kirschner, 1930).

b. Necrosis and necrobiosis. The healing process in both open and closed wounds is primarily dependent on the depth to which the Joule's heat acts. In the coagulative type of current there occur coagulation necroses which extend to variable depths, whereas with the cutting current these are proportionately less extensive. Since the necrotic layer merges gradually into unaffected tissue, there is always an intermediate zone of necrobiotic tissue. Hence it is that the healing course of electrosurgical wounds will be determined primarily by the extent and degree of retention of the necrotic and thermally injured tissues. The extent of influence of the cutting current is dependent on the conductivity of the several tissues which may be arranged in the following series: blood > nerve > muscle > skin > liver > lung > brain > fat > bone (Wildermuth, 1911; Bucky, 1929). The perfection of electrosurgical apparatus now permits the cutting of tissue with the coagulation-spread reduced to a minimum. But Bauer (1931) has also shown that even those cuts with the least coagulative effect produce a necrobiotic border zone after 24 hours' time; such wounds never exhibit a cleft-like space but a coagulation furrow, of greater or less breadth, produced by the burning-out of tissue.

c. Relation of necroses to repair. The layer of coagulated tissue bordering an incision prevents the provisional sticking together of the sides of the wound. It represents material that has to be removed, either by casting out as a whole or by resorption, before proper reparation can proceed (Bauer, 1931; Hauberisser, 1931; Dressel, 1932). The conditions become still less favorable when the prereparative period is prolonged by the displacement to deeper levels of coagulation necroses, as may occur in sewing-up of the wound. In such instances the coagulated tissue, together with its bordering necrobiotic zone, becomes encapsulated through granulation tissue in the same way as does any other foreign body (Kuntzen and Vogel, 1931; v. Seemen, 1932). In the course of time it becomes resorbed through the operation of shrivelling and liquefaction. After months v. Seemen could still demonstrate such degenerative effects in skeletal muscle. It is manifest that a sunken sequestrum of this type can seriously disturb the progress of wound healing, and especially if it becomes cast out. From the foregoing considerations it is also apparent that primary wound healing following electrical incisions is at a disadvantage in comparison to the more straightforward course of wounds produced with the scalpel.

With open coagulation wounds, on the other hand, the marked, surrounding hyperemia leads to the prompt casting-off of the scab, and of the associated necrobiotic tissue, and to the development of vigorous granulation tissue and a resultant rapid cicatrization.

d. Other peculiarities. The open, coagulated wound presents some additional distinctive features. The base of the broad and thick coagulation scab is firmly fixed. In contrast to the usual scab of wounds and burns it is permeable to fluids so that a retention of secretions beneath the scab does not occur. On the other hand, the scab is not permeable to bacteria. It also furnishes a strong stimulus to regeneration.

The tissues definitely injured by the electric current show a characteristic drawing-out of cells and their nuclei into elongated elements arranged more or less parallel with the direction of the cut. This is seen in epidermis where the basal and prickle cells are involved but is best exhibited in muscle and soft connective tissue (Bauer, 1931; Ellis, 1933). A similar phenomenon has been described radiating from areas of the skin involved in fatal electrical injuries (Kawamura, 1921).

Electrically produced skin wounds of dogs showed primary union in only 60 per cent of the cases in comparison to 97.5 per cent of primary union in scalpel wounds (Ellis, 1931). Even in those animals in

which primary union was accomplished, the wound, as tested by its tensile strength, was somewhat weaker until the twenty-fourth day of healing. However, electrical wounds made in the stomach wall and in muscle were at no important disadvantage in primary healing in comparison to those produced with the scalpel.

The coagulative effect on the wound surfaces at the moment of tissue severance results in the instantaneous closing-off of capillaries, lymph channels and tissue spaces. This brings about an effective separation of the body as a whole from the wound, so that absorption of the products of the destructive phase of healing is reduced to a minimum, as may be proved by the evidence of their limited presence in blood and urine (v. Seemen, 1931). Correlative proof of this isolation is furnished by Zschau (1931) who has shown that curare poisoning is suppressed when this drug is applied to electrosurgical wounds whereas the absorption is high from ordinary scalpel wounds. Moreover, v. Seemen reports a markedly delayed resorption of tetanus toxin under similar conditions. It is obvious that such isolation of electrosurgical wounds from the body proper permits the execution of extensive surgical attacks with a high degree of protection to the individual. The fact that this sparing action by isolation continues postoperatively applies not only to the absorption of katabolic products but also to the spreading of bacteria and the transportation of cellular elements, including those of tumors (Zschau, 1931).

In addition, shock phenomena are largely eliminated (v. Seemen, 1931; Bauer, 1932). In fact, v. Seemen (1931; 1932) reports that during electrosurgical operations on the human subject he has observed only a slight and temporary drop in blood pressure, little or no variation of the pulse and no disturbance of the electrocardiographic record; direct measurements of the carotid blood pressure on the dog during extensive electrocoagulation of the soft parts likewise gave no substantial variation.

III. Mensuration. The course of normal wound healing has been subjected to mathematical analyses by a number of investigators. Several component phases of the general phenomenon can be identified and formulae derived which fit the total curve. Since there are definite mathematical relationships between such factors as the size and age of the wound, the age of the patient, etc., the rate of healing becomes determinable and its course and termination predictable.

The areas of skin wounds can be traced on cellophane and measured by planimeter or weighing methods. Carrel (1910) and Carrel and

Hartmann (1916) have thus studied sterile skin wounds on experimental animals and on man, and have been able to plot the diminishing wound area during epithelial repair. These data apply to sizeable lesions on loose skin in which an appreciable area (more than 1–1.5 cm.) of skin has been lost. As already pointed out (p. 350), epidermization starts sooner and contraction ends later in firmly attached skin such as is found on the ears (Loeb, 1898; Burrows, 1924), although some differences doubtless occur even in the latter, less common locations. Carrel's data, given with special quantitative reference to the dog, can be summarized into four periods, as follows:

a. A latent period, during which the wound remains practically the same size, follows the injury. Its duration is variable, lasting between one or two days and four or five days. The length of this period, however, can be readily prolonged or shortened by various means, as will be explained in a later paragraph.

b. A period of contraction, which is very active at first and then slows progressively to a standstill. The rate of contraction is directly proportional to the size of the wound and not to its age, so that wounds of different initial sizes tend to equalize as healing progresses; this conclusion was also reached independently by Spain and Loeb (1916). The rate becomes reduced to zero when the edges of the wound are about 10 to 15 mm. apart. In wounds larger than 40 mm. the contraction often stops when the exposed wound is reduced to 20 or 25 mm. The phenomenon of contraction plays a most important rôle in the healing of medium- or large-sized wounds; on the other hand, it is of little or no value in small wounds of 15 mm. or less, since epithelial regeneration can easily accomplish repair unaided.

c. A period of epidermization, which not only follows contraction but, indeed, brings it to an end. However, the two processes often overlap and proceed as concurrent phenomena. The beginning of epidermal regeneration depends also on the size of the wound and not on its age; it makes a tardier appearance in large wounds and the growth is exceedingly slow if the distance to be covered is more than 12 to 15 mm.; in fact, epithelization actually comes to a halt if the edges of the wound are still some 20 to 25 mm. apart when contraction has reached its limit. On the other hand, wounds less than 10 mm. wide are covered rapidly. Hence it follows that the rate of epidermization is inversely proportional to the size of the wound; in accordance with this principle the rate actually becomes maximum toward the end of healing when the edges of the wound are close together.

d. A period of cicatrization, which extends over a relatively long time, ends the healing process. As the result of the variable effect of contraction already mentioned, the scar is proportionately smaller in the larger wounds. When the original wound is small enough (10–12 mm.) so that contraction never appears, then wound and scar are of the same size. Following epithelial healing there is an enlargement of the scar by spreading. In general, an infection introduced during any period tends to prolong both that period and the subsequent ones as well, unless the infection be successfully routed in the meantime.

As the result of Carrel's analysis of the stages of healing, which shows that the diminution in size of an aseptic wound is regular and geometric, du Noüy (1916a, b) developed an empirical formula by which the rate of healing in any given wound can be determined, as soon as accurate measurements are obtainable, and its course and termination predicted. Under given conditions of asepsis and dressing it was found that the area cicatrized each day is directly proportional to the size of the wound, to the square root of its age, and to the relation between the rate of cicatrization and the square root of the age of the wound at the time of observation. The age of the wound in itself seems to have no influence on the rate of wound healing. The use of age in the formula is merely because wound size can be expressed conveniently in terms of time which then becomes one of the factors of its dimensions. The age of the patient does bear a proportional relation to the area of the wound and to a constant which characterizes each wound and must be determined in each case. In 1932 du Noüy was able to establish a constant of physiologic activity expressed in terms of the patient's age alone. The equation of du Noüy proved of practical value and was in general accord with the observed facts, as Ingebrigtsen (1918), Tuffier and Desmarres (1918) and others found. Nevertheless, some deviations were noted by these observers, and other writers have also directed certain criticisms against it. Already in 1917 de Beaujeu had suggested an improvement in this formula and in 1919 du Noüy presented new equations of a more purely mathematical nature by which the rate of contraction alone and the summated effect of contraction and epidermization might both be expressed. Even this more perfect expression has not been without criticism (Brownlee, 1924), but perhaps it furnishes the best approximation of the predictable course of a phenomenon notable for its complexity and inherent variables. The formula proposed by Brownlee is computed for circular wounds alone and so is not of general utility.

Still later Carrel and du Noüy (1921) returned to an intensive study of the latent period. In the dog this lasted five to seven days (under the conditions of experimentation imposed) and then ended abruptly. The contraction period followed at maximum velocity from the very start, so that du Noüy's formula applies to the beginning of contraction as well as to its subsequent course. Nevertheless, the length of the latent period can be influenced in various ways. If the wound be protected by a non-irritating dressing contraction is delayed for weeks, whereas the local application of irritants, such as turpentine, chick-embryo pulp or staphylococci, reduces the duration of the latent period to two days (Carrel, 1921). Similarly Clark (1919) found that protein diet practically eliminates the latent period, while fat-fed animals, due to the presence of a typical latent period, healed their wounds some five days slower. None of the other periods of healing was influenced by diet. Somewhat different are the conclusions of Harvey and Howes (1930) who observed no shortening of the latent period of fibroplasia in stomach wounds of the rat subjected to high protein diet. However, they did find an increase in the velocity of fibroplastic healing when once the process was started, so that repair was significantly shortened.

But these are not the only attempts to plot and predict the course of wound healing. Another empirical formula, entirely different and much simpler than that of du Noüy was proposed by Lumière (1917, 1918). This author concluded: that the speed of cicatrization is essentially equal in animals of the same age, condition and kind; that the absolute speed is constant throughout the healing period; and that the total time of cicatrization is approximately proportional to the greatest breadth of the wound. Hence the total healing time in days can be determined by dividing the greatest breadth by a constant which for man is about 0.95. The agreement between the observed and calculated results is remarkably good in the examples given. A reply to certain criticisms of Amar (1918) was made by Lumière in a later publication (1918).

Another formula of the theoretical, mathematical type has been propounded by Fauré-Fremiet and Vlès (1919) who have been impressed with the idea that the laws governing the special phenomena of cicatrization ought to be consistent with the broader laws governing growth as a whole. In particular have they been influenced by the equations of Robertson (1923) who interprets growth of the organism and its parts as an expression of a monomolecular, autocatalytic reaction.

Having derived formulae based on the same fundamental considerations as those of Robertson, these authors have obtained a precise agreement with two curves as calculated by the du Noüy formula.

The latest attempt at formula compounding has been presented by Brummelkamp (1929) who believes his calculations fit Carrel's curves of actual cases better than do the values computed by the du Noüy formula.

A different kind of attack on the problem of healing rate has been made by Howes, Sooy and Harvey (1929) who discovered that the rate of cicatrization of a simple, incised wound may be followed by testing its returning tensile strength. In essence this procedure merely measures the progress of fibroplasia, which is interpreted by them as the basis of contraction, and avoids the epithelization factor. The results on wounds of the skin, fascia, muscles and stomach of the dog show a latent period of four to six days followed by a phase of fibroplasia during which the wound gains strength rapidly at first and then more slowly as the maximum strength is approached. The latter point is attained in 10 to 14 days. The curves obtained are of the same general nature as those of Carrel, and both have features comparable to the autocatalytic curve of growth and some chemical reactions (*cf.* Brody, 1927; Robertson, 1923). Repetition of this type of experiment by Iwashima (1930) has resulted in a general confirmation.

Harvey (1929) also discovered that the latent period of approximately four days was a period before growth of the fibroblasts becomes appreciable in terms of function. The velocity of fibroblastic growth he found to start abruptly and diminish progressively in rate, as Carrel (1910) had previously determined for contraction. Following the lead of Clark (1919), Harvey and Howes (1930) showed that high protein diet did not lessen the latent period in wounds of the stomach of the rat, but did increase the velocity of fibroplasia once started and brought it to an earlier end. Furthermore, the rate of return of tensile strength in adult rats was not affected by complete or partial starvation, although a half-adequate diet did result in considerable retardation in young rats (Howes, Briggs, Shea and Harvey, 1933). Just as du Noüy (1916b, 1932) had shown a faster rate in the wound healing abilities of young individuals, so Howes and Harvey (1932) found in young rats an earlier onset of fibroplasia, a lessened retardation, and an earlier termination of the process, although the rate itself was not increased.

IV. PHYSICO-CHEMICAL RELATIONS. In comparison to the structural features of wound healing, which are now largely worked out, except

as they are waiting on new technical methods of attack, the chemical and especially metabolic phenomena are but partially known. The investigations of recent years, however, have done considerable toward correcting such deficiencies, and the information now at hand is definitely useful in rounding out and explaining the microscopical picture of wound healing. Some of the physico-chemical phenomena of inflammation and wound healing have been reviewed by Häbler (1928) and Tammann (1933).

1. Loss of Water, Heat and Carbon Dioxide. Apparently there are no direct measurements of the loss of water and heat from open or granulating wounds. Some data are available on the exposed serous surfaces of the rabbit's intestine where A. W. Fischer (1932) found the loss per square centimeter in one hour at 26°C. amounted to only 0.15 gram of water. On a model intended to reproduce the conditions in the intestine there was a loss of radiant heat amounting to 48 gram calories per square centimeter per hour, while the loss by evaporation was 85 gram calories. All these values are too small to constitute significant losses.

Reimers and Winkler (1933) have measured the loss of carbon dioxide from uninfected wound surfaces. The average loss per minute from each square centimeter of surface amounted to 0.035 cc. This is some thirty times the amount given off from the uninjured skin of man.

2. Fluid Content of Wound Tissues. The tissues of cleft-like and gaping wounds contain more than the normal amount of fluid. Tammann Blümel and Roese (1933) have measured the water content of aseptic skin transplants in the rabbit. An increase occurs within three hours, whereas the maximum hydration is attained by the end of the first day. A gradual decline then follows as the healing process advances, until the normal content is reached again in the third week. Since, on the other hand, dying transplants experience a progressive water loss these relations are somewhat indicative of wound prognosis. How an approach to the more sol-like condition of fetal tissues is brought about in the wound is not entirely understood. Both a purely physical absorption of water through swelling and a chemical change of the colloidal micellae by a taking-on of water through hydrolysis are possible. Apparently both processes take place (Häbler, 1928).

3. Electrolytes and Osmotic Pressure. Increase in the water content of the wound territory brings a concomitant influx of electrolytes. Andreesen and Tammann (1933) were able to collect the meager tissue-fluid of aseptic wounds by a special method and found that the potassium and calcium content was increased in comparison to blood serum,

but that sodium and chlorine diminished. Similar results were obtained both in lower mammals and in man. In the quantitative determinations of Tammann, Blümel and Roese (1933) on healing transplants there was an increase in sodium, potassium and chlorine. Häbler (1929) found that the greatest individual storage was of potassium. Whereas this amounted to 12 to 16 mgm. per cent of the normal plasma, it doubled in sero-purulent and sero-fibrinous exudates and increased to 51 to 205 mgm. per cent in purulent exudates.

Andreesen and Tammann (1933) were also able to measure the freezing point of the limited secretion of aseptic wounds. This was regularly lower than the corresponding value for serum. Similarly the osmotic hypertonia of the more abundant exudate of pyogenic wounds has been measured by Ritter (1902, 1905) and that of furuncles by Schade (1923). In comparison to a $\Delta = -0.55$ to -0.58 for blood these investigators obtained values of -0.6 to -1.4. In the inflammatory zone of manifest edema toward the periphery there were decreasing values of -0.76 to -0.58. These amounts represent the excess osmotic hypertonia for which the body has not been able to compensate.

4. Electric Potentials. It is to be expected in the production of pronounced changes in the colloidal condition of wound tissues and in the creation of many active surfaces that differences in electrical potential would appear. Especially is this true of granulating wounds. Melchior and Rahm (1918) discovered a constant electric current in granulating wounds whereby the granulation tissue was positively charged with respect to the surrounding skin. This they designated an "action current of the granulating wound." Beck (1922) took issue with this interpretation and held that the potential difference measured by the former workers was merely an action current generated by the cutaneous glands. Since, however, Melchior and Rahm (1923) have answered these criticisms, and especially since their findings have been confirmed by Herzen and Nissnjewitsch (1925), there would seem to be no further doubt as to the existence of such a current in the wound tissue. The latter authors found the strength of the current bore a direct relation to the exuberance of granulation tissue. An especially strong potential difference was measured in the neighborhood of necroses; in epithelizing granulations they were weak or lacking. According to their interpretation the current depends on the changed conditions of dispersion and concentration of the colloidal solution in injured and regenerating tissues, as well as upon surface tension phenomena.

5. Mitogenetic Radiation. Until recently there has been but little

evidence that mitosis can be initiated or accelerated under physiological conditions except through heat. In 1923, however, Gurwitsch believed he had demonstrated the existence of "mitogenetic rays" (really identical with ultraviolet light) which are given off by cells or cell extracts and will induce division in other cells (*cf.* p. 334). A comprehensive review of this subject, with special emphasis on the positive results has been furnished by Gurwitsch (1932). For criticisms, negative experimental results, and expressions of scepticism as to the existence of radiations of the kind and effectiveness described reference should be made to contributions by Taylor and Harvey (1931), Richards and Taylor (1932), Gray and Quellet (1932), Schreiber (1933), and Helff (1934). The specific relation of mitogenetic radiation to wound healing has been investigated by Bromley (1931), Blacher, *et al.* (1932), and Samarajew (1934) who claim positive results, and by Helff (1934) who failed to confirm such findings.

6. Hydrogen-Ion Concentration of Wounds. As a result of the stasis in the vessels there results an accumulation of carbon dioxide which brings about a local acidity. The resulting oxygen-want also effects an alteration of the cellular metabolism in which organic acids (lactic; butyric; etc.) are elaborated; since these are not transported away efficiently the local acidity is correspondingly increased to a further degree.

Girgolaff (1924) has used the subcutaneous electrode method of gas chain measurement, as developed by Schade, Neukirch and Halpert (1921), for determining the acidity of inflammatory exudates. In aseptic, stitched wounds of the rabbit, values of pH 6.71 were obtained at two hours, pH 6.32 at 24 hours, pH 6.71 at 9 days and pH 7.05 at 14 days. These results are to be compared with a slightly alkaline reaction (pH 7.1–7.3) measured in normal tissues by himself and others. With similar technique Tammann (1931) found a maximum pH of 5.6 to 6.0 after three hours in transplants of the rabbit.

Rohde (1927), employing indicators, obtained values of pH 6.4 to 7.4 for the tissue fluid of healthy, fresh granulations. In chronically inflamed granulation tissue the pH values were less acid, with a preference toward the neutral range (pH 6.8–7.1) or toward weak alkalinity pH 7.4). The exudate of the healthy granulations gave acid reactions, even to pH 5.8, whereas the inflamed type had a maximum acidity of only pH 6.4, in half of the trials was neutral, and in isolated cases passed over into alkalinity (pH 7.8). In dormant, edematous (and also in abnormally dry) granulations Rohde also found a value of pH

6.4, while occasionally there was a strong alkalinity to pH 8.5. Also with the colorimetric method Fontaine and Jung (1928) found the early exudate of open wounds in rabbits to be practically neutral (pH 7.07). It should be noted, however, that the methods employed by these investigators could not take cognizance of errors introduced by the evaporation of carbon dioxide.

With a far superior technique through the use of quinhydrone electrodes, Schade and Claussen (1926) found a strong alkalinity on the surface of granulating wounds; the maximum measured was pH 8.3. The cause of this considerable local alkalinity lay in the evaporation of carbonic acid, and after conducting 5.6 vol. per cent of carbon dioxide over the wound surface the pH value passed back again to the acid side. Furthermore, in fresh wounds made by cutting the healthy skin, the secondary alkalinity of the surface secretion (as the result of evaporation of carbon dioxide) was proved; after 30 minutes it registered pH 8.3. Nevertheless, as might be expected, the acidity increased at progressively deeper levels. In a later investigation Schade, Beck and Reimers (1930) have dealt especially with the problem of carbon dioxide evaporation and the sources of error contained therein. Confirming the older determinations of Schade and Claussen, they obtained pH 7.13 to 7.77 on the surface of granulating wounds. Only in deep pockets under scabs did they find acid values (pH 6.70).

Schmidtmann (1927) and Schmidtmann and Matthes (1927) have attempted to study the reactions of individual cells and cell groups by introducing indicators with the micromanipulator apparatus. Though only relative rather than absolute values were obtained, it is of interest to learn that the fibroblasts of rabbit granulations are more acid than the lymphocytes and plasma cells.

7. *Influence of Diet on pH.* Schade, Beck and Reimers (1930) have followed experiments on dogs for weeks, with the loss of carbon dioxide from the surface eliminated. Perforated, hollow glass spheres were sunk in the tissues and the wound secretions collected and analyzed. On the dog the influence of acid and alkaline diet was investigated. A sure change of the pH values could not be established, but there was a definite increase of the carbon dioxide-binding power under alkaline diet and a corresponding decrease under acid diet. Under acid diet the quantity of wound secretion was regularly and considerably scantier than after alkaline diet. This they believe was due to an increasing of the oncotic pressure in the compensatory acidosis of blood whereby there was a marked swelling of blood corpuscles and resorption of tissue

fluid in the capillary region. In blood alkalosis, the opposite occurs and there is a favoring of exudation.

From the clinical side Herrmannsdorfer (1927), supported by Sauerbruch (1924), has shown clearly that the kind of food eaten influences the course of wound healing, as did Clark (1919) and Harvey and Howes (1930) on experimental animals. Herrmannsdorfer seems to have established the markedly favorable action of an acid diet. Especially does the bacterial flora then decrease, whereas under alkaline diet the wound swarms with bacteria and often becomes foul smelling, while young epithelium is cast off. Although some divergent opinion has been expressed concerning the effect of the Sauerbruch-Herrmannsdorfer dietary regimen on the acid-base equilibrium (Beckman, 1931), the main contentions of Herrmannsdorfer have been confirmed by Reimers and Winkler (1933). These investigators produced an acidosis in dogs through the administration of ammonium chloride and found a concomitant shortening of the course of wound healing.

Schulze (1933) has emphasized the relation between local acidity and the presence of a strong potassium reaction, on the one hand, and neutrality or slight alkalinity and a strong calcium reaction, on the other. During the destructive phase of wounds produced in dogs he found this potassium excess, whereas calcium preponderated during the period of rebuilding. These findings are correlated with those of Balint and Weiss who record that in rabbits previously made "acid" by diet the destructive phase was accentuated while alkalinized animals exhibited accelerated reconstructive activities.

8. Effects of Acidity on Wounds. Both the structural and metabolic changes are qualitatively similar in inflammation, wound healing and transplantation. From the foregoing resumé it seems safely established that the reaction in the wound area, especially in the earlier stages, is pushed toward the acid side. Through this local acidity various concomitant conditions are induced:

a. Enzymic autolysis. The action of autolytic enzymes in connective tissues is definitely dependent on the acidity of the reaction (v. Gaza and Brandi, 1927b). For example, when the autolysis of a sarcoma *in vitro* is measured by the amount of nitrogen in the waste product, there are proved to be two opposing optima for enzymic activity. One is in strong acidity (pH 3.9) and the other at a considerable alkaline level (pH 8.6). Still a third optimum lies intermediate in position (pH 6.4) which these authors believe to be the optimum point for the most efficient summated action of all proteases. An increased activity

of the proteolytic enzymes is possible in the wound area since the existing pH value lies in the neighborhood of their optimum.

b. Mitogenetic radiation. The intensity of the ultraviolet rays, claimed to emanate from the products of tissue proteolysis and of significance as stimulators of cell proliferation, is also said to vary with the pH values of the local tissues. With increasing acidity the radiation becomes more intense (Blacher and Bromley, 1931).

c. Capillary dilatation and its results. Investigations carried out with an exact maintenance of hydrogen-ion concentration and buffering show that limited acidity induces a considerable dilatation of capillaries. Fleisch (1921) found that the addition of 0.5 to 5.0 vol. per cent of carbon dioxide to Ringer's solution, or of n/1000 hydrochloric acid, brought about a plain vasodilatation in the frog. Also in the frog the maximum dilatation occurs between pH 5.0 and 7.0 whereas under more acid or alkaline conditions capillary contraction enters (Atzler and Lehmann, 1921).

In the rabbit, as well, a lessening of the hydrogen-ion concentration caused vasoconstriction, while an increase up to about pH 4.5 induced vasodilatation, above which point constriction again took place (Atzler and Lehmann, 1922). The results of these latter investigators are all the more significant since the limits determined by them lie within the measured pH range of the wound tissues.

A mechanical factor in dilatation is furnished by the elastic contraction of severed connective-tissue bundles, which thereby undergo a volume increase and exert a compression in the vessels. This leads to stasis of the blood stream. Moreover, trauma to the nerve supply and muscle of blood vessels may also result in expansion of the capillaries.

Concurrent with capillary dilatation goes a slowing of the blood flow which effects a separation of the blood elements in a purely physical manner. The lighter leucocytes take a peripheral position along the capillary wall. Their adhesion to the endothelial membrane is apparently also a physical phenomenon due to a lowered surface tension, but the cause of such reduction is not clear.

Gräff (1922) showed that leucocytes were attracted by organic acids and assumed that this effect is due to the influence of the H-ion, whereas Siegel (1925) found the same emigration from the capillaries of the frog's web in regions treated with acids and alkalies as occurred in control, untreated spots. However, Feringa (1924) was led to conclude that the diapedesis of leucocytes in rabbits depends largely on a non-

specific, chemotactic stimulus exerted through the hydrogen hyperionia in the neighboring tissues. But there are other factors involved besides chemotaxis; specifically may be mentioned surface tension phenomena (Häbler and Weber, 1930) and the electromotive forces at play in the tissues (Abramson, 1927). The latter investigator found the surface of injured tissue to be negative with respect to uninjured tissue, so that the leucocytes which *in vitro* wander cataphoretically to the negative pole would also tend to move from the capillaries toward the site of injury. In passing it might be mentioned that the existence of stomata in the capillary wall through which leucocytes might pass, although once believed, is not accepted today; in fact such are not necessary since the colloidal loosening of the endothelium through H-hyperionia makes direct passage possible. The favorable significance of acidity for phagocytic activities has been treated by Feringa (1924).

d. Transmission of plasma colloids. The capillary wall, swollen through acidity, becomes permeable to plasma colloids in the same way that occurs in acute inflammation. The associated changes actually taking place in these colloidal membranes are even less clear in the living endothelium than in physical models. Opposed to Oswald (1910) who believes in a heightened permeability to colloids of the endothelial membrane proper is the view of v. Gaza (1918a) who assumes that especially does the intercellular cement become more swollen and permeable than the endothelial cells themselves; the latter respond less in these directions because of their lipoidal content. Oswald (1910) has shown that the proteins of the plasma are arranged in an order of permeability according to the following series: albumin > globulin > fibrinogen. A secretory activity on the part of the capillary wall has not been proved; according to the investigations of Schade and his co-workers (1926) this is at best improbable. It would seem that the egress of colloidal substances is sufficiently explained by the increased vascular pressure and heightened permeability of the endothelial wall, both as the result of dilatation and through colloidal alterations brought about by the local acidity.

e. Swelling of tissue colloids. Studies on the swelling phenomena of tissue colloids have been made by several workers. Some, but not all, of these phenomena observed in wounds are related directly to the acidity of the wound site.

Schade (1913) and Schade and Menschel (1923) found that the ground substance of the umbilical cord (mucous tissue) and the collagen

of tendon behave in an opposite manner toward H- and OH-ions. Mucous tissue swells in an alkaline medium and shrinks in an acid medium; tendon behaves in a reverse manner. In intravital experiments they obtained minimal swelling of normal human skin at pH 6.0; as a matter of fact, at this concentration there was a slight shrinkage. They further proved that mechanical as well as oncotic pressure is significant, since lowering of the mechanical pressure brings about an increased swelling of the connective tissue. Hence the release of tension and pressure through the severing and retraction of tissues of the wound site would of itself favor connective-tissue swelling.

Tammann (1931a) and Tammann, Blümel and Roese (1933) on healing transplants showed the relation of swelling to buffered phosphate mixtures in comparison to that for normal skin. In the rabbit the swelling in transplants is increased, with the minimum swelling more on the acid side than the corresponding point (pH 6.9) for normal skin. As healing advanced, these differences were progressively equalized.

In healthy skin the swelling toward anions and kations follows the well known Hofmeister's series for the action of the ions of neutral salts in precipitating proteins. On the other hand, wound tissue exhibits, as v. Gaza and Wessel (1923) also demonstrated, marked departures. In inflamed wounds (with increased acidity) there is a reversal toward the kations sodium and calcium in comparison to normal tissue, so that sodium produces shrinkage while calcium (and also magnesium) swells strongly. The chlorine anion hinders the swelling of inflamed skin or arrests it completely, whereas the sulphate radical produces the opposite effect. In contrast to these results Tammann (1933) has investigated by the same method the swelling phenomena in aseptically healing transplants of the rabbit. The normal skin followed regularly the Hofmeister's series, and the transplants also showed only quantitative, rather than qualitative, departures. These results would seem to indicate a clear difference between aseptic and infected wounds. Osmotic influences play only a subordinate rôle in such swelling phenomena, since Tammann was able to show that increased swelling occurs even though the transplant is essentially richer in water than the normal skin.

Fibrin exhibits a behavior essentially comparable to that of other tissue colloids. But Wöhlisch (1924) found a minimum of swelling between pH 6.0 and 6.3 which is the acidity of the early primary healing wound (Girgolaff, 1924). However, the acidity recedes as healing progresses; this is favorable to the swelling and solution of the fibrogel.

f. Pain. The relation of pain in aseptic wounds to local acidity has been studied by v. Gaza and Brandi (1926) who have been confirmed by Häbler and Hummel (1928). The injection of buffered acid phosphate solution into the skin is painful for a short time until the normal tissue reaction is restored by the buffering action of the blood. Buffered alkaline phosphate solution arouses no painful sensation. Pain began at pH 7.2 and increased progressively until at pH 5.9 the investigators on self-conducted experiments found the pain unbearable. Although in aseptic, healing wounds a similar acid reaction can scarcely arise, or almost momentarily is modified through the buffering of tissue fluids, yet in inflamed wounds there is the possibility of strong pain on the basis of local acidity. Indeed, v. Gaza and Brandi (1927a) have been able to relieve the pain from abscesses, ulcers and allied conditions by the injection of buffered alkaline phosphate solution. That other factors besides acidity play a part in the causation of pain will be apparent in the fuller discussion of this subject on p. 375.

9. Metabolism of Wound Tissues. The metabolic rate of normal human connective tissue, although very low, is still appreciable. Groll (1927) proved by gas analysis that tissue cells of the mouse's ear react to an inflammatory stimulus by increased oxygen consumption, which is a response of the cells themselves and not a secondary result of hyperemia. Using Warburg's (1926) method, Neuhaus (1929) investigated the respiration and glycolysis of granulation tissue in a series of animals and found that the glycolytic metabolism follows the following order: guinea pig < dog < rabbit. In the rat the level of metabolism equalled that exhibited by malignant tumors.

v. Gaza and Gissel (1932) have likewise used Warburg's method to study respiration and anerobic glycolysis in aseptic wounds of the human skin. The results of both processes follow the same general course. Shortly after the infliction of the wound there enters a rapid fall in the metabolism of the wound area which corresponds to the destructive phase in the events of wound healing. The ensuing metabolic rise coincides with the arrival of amoeboid cells of the blood and connective tissue, and characterizes the assimilative phase. Finally, as a third phase, follows the decline of wound metabolism to the normal level. They ascribe the destructive period of wound metabolism to the action of katheptic enzymes, which through their activator, zoökinase, become set into action in a weak acid environment. The presence of zoökinase (reduced glutathion) in the wound tissues could be demonstrated in alcoholic extracts. In healthy tissue, glutathion occurs almost solely in the oxidized form, but in conditions of oxygen-want

and feeble acidity, as holds in the wound area, it assumes the reduced form and works as an activator of tissue proteolysis.

In the same manner, v. Gaza, Gerlach and Gissel (1932) have investigated the metabolism of the Achilles tendon of the dog under aseptic conditions. This was selected because of its simple structure, low cell content and slow metabolism. The course of metabolism was similar to that in the wounds of the human skin, already described, and showed good correlation with the morphological picture of regeneration.

10. Effect of Wounds on the Body as a Whole. The presence of a significant wound is reflected on certain more general conditions and activities of the body. Large losses of blood are replaced as to volume by the blood stream taking up fluid from the tissues. This compensation effects a dilution, a consequent decrease in viscosity, and a lessening of the buffering power. Within the first day there results a shift in the blood toward alkalinity, with an increase of the alkali reserve, and an increase in the blood sugar (Wymer, 1925).

Changes in the red blood picture are primarily dependent on the extent of hemorrhage, and are not especially consistent. The white cells immediately after an operation may show a leucocytosis as an expression of the acidosis, but it is only after the resorption of the degenerative products of the wound area, some 12 hours or more later, that a stimulation leucocytosis appears (Löhr, 1922). The blood platelets were found to diminish in number (up to 50 per cent) during the first five postoperative days, whereupon an increase then occurs which rises between the eighth to eleventh days to some 50 per cent above normal (H. Hueck, 1925). The changes in the physico-chemical blood constants following operations have been investigated by numerous workers who have also pointed out various correlated relations. Such determinations are naturally complicated by the effects of narcosis as well. For these data and interpretations reference may be made to the publications of Löhr (1923), Wymer (1925), Bürger and Grauhan (1927), Boshamer (1928), Heusser (1928), v. Seemen and Binswanger (1928), and Tönnis (1929).

Schneider and Straaten (1928) have studied the general metabolic rise through measurements of the basal metabolism. In the destructive phase of wound healing, utilizing nonfebrile individuals with severe wounds and burns, a considerable oxidative increase was determined, while in the constructive period of repair the higher basal metabolic rate no longer obtained.

11. Physico-chemical Basis of Inflammatory Symptoms. The cardinal symptoms of all inflammation, including that associated with wound healing, are *rubor*, *tumor*, *calor*, *dolor*, and *functio laesa*. The conditions believed to constitute a functional basis for these phenomena can be summarized as follows:

a. Rubor. The redness of inflammation is due fundamentally to hyperemia which, as the first recognizable effect, comes as the result of a series of happenings. Schmidtmann and Matthes (1927) have shown that there is an early increase in the permeability of the surface of the cells of inflamed connective tissue. It is possible that this results in the loss of potassium from the cytoplasm (Häbler, 1928) which would then upset the isoionic balance of the sodium-potassium-calcium content of the tissue fluid, and especially the ratio of calcium to potassium (Schulze, 1933). In any event, when cells are injured or inflammation is set up the potassium increases (Schade, Claussen, Häbler, *et al.*, 1926; Tutkewitsch, 1927) and can reach high values (Häbler, 1929). In this connection it is significant that potassium is potent in increasing the irritability of nerves (Jahn, 1924), while the ratio of potassium to calcium is of particular significance to the tonus of the sympathetic nervous system (Zondek, 1922, 1925). It must be admitted, however, that a clear understanding of how these electrolytes act on the arterioles, capillaries and venules is not yet established (Zondek, 1927). But the fact that potassium injected into the wound promotes hyperemia (Schück, 1926; Häbler and Hummel, 1928) would seem to aid the general hypothesis just advanced.

Injury to the tissue cells of an inflammatory focus also liberates enzymes which lead to an elevation of the local tissue metabolism. Through this increased activity there comes a concentration of metabolic end products (organic acids and carbon dioxide) which produce a local acidity. Such a local concentration of H-ions exerts a fundamental influence on vascular tonus, as various workers have shown. Especially significant are the modern investigations of Fleisch (1921) and of Atzler and Lehmann (1921) in which due attention was paid to pH and buffering phenomena. Fleisch proved that the addition of 0.5 to 5.0 vol. per cent of carbon dioxide to Ringer's solution produced a plain vasodilatation in the frog, as did also n/1000 hydrochloric acid. The greatest dilatation in the frog is obtained in the range of pH 5.0 to 7.0, with an optimum at pH 5.7. Greater acidity induces vasoconstriction, as does an alkalinity beyond pH 7.0. Experiments on the rabbit also show vasodilatation up to about pH 4.5 (Atzler and Leh-

man, 1922). But since it has been found that a pH on the acid side is characteristic of inflammation (p. 365), it seems unquestionable that the local acidity plays a prominent part in producing the the inflammatory hyperemia. Häbler (1928) believes it probable that the same factor is operative even in the initial hyperemia at the beginning of inflammation, although no measurements of pH values at that time have as yet been made.

b. Tumor. Another effect of the increase in H-ions is the swelling of protoplasmic colloids (Hamburger, 1904). With the increase in swelling goes a decrease in the firmness of colloids; this "loosening" effect brings about an increased permeability of the capillary endothelium by which the ultramicroscopic pores of this membrane enlarge in size. Normally the endothelium serves as an ultrafilter permeable chiefly to water and solids in true solution. Now, however, the heightened permeability permits the passage of the blood proteins into the surrounding tissues in the same manner that the colloidal dye, Congo red, when injected intravenously, in contrast to its normal behavior, appears promptly in the inflammatory zone (Hoff and Leuwer, 1926). This inflammatory exudate is chemically different from the non-inflammatory transudate. In the transudate, albumin is the favored protein constituent, whereas in the exudate other proteins are featured as well, including a much greater quantity of globulin. According to Oswald (1910) the several proteins leaving the vessels during inflammation can be arranged in the following series (based on their quantity and ease of passage): albumin > euglobulin > pseudoglobulin > fibrinogen. This transfer stands in direct relation to their lessened viscosities; it follows the diffusibility sequence exhibited generally with respect to colloidal membranes (Bechold, 1912).

According to Oswald (1910) the increased permeability is a function of the endothelial cells proper, whereas v. Gaza (1918a) concludes that the intercellular cement is the responsible factor. The latter worker holds that the lipoidal content of the endothelial cells themselves offers considerable resistance to swelling. He infers that since corpuscular elements pass out intercellularly it is probable that the exudate takes the same route, since otherwise it is necessary to postulate a double pathway.

The progressive relation of increased acidity to heightened inflammation and to colloidal swelling permits the foregoing inferences as to the relation of H-ion concentration to capillary permeability. It is possible that the acidity effects a lowering of the viscosity (or some other col-

loidal changes) in the proteins themselves; yet, on the other hand, the increased permeability of the capillary wall is not sufficient of itself to explain the passage of the inflammatory exudate. Hence there must be some driving force which causes the passage of the nonliving proteins through the membrane. A secretory activity on the part of the endothelial cells is not only unproved, but is rejected by Schade, Claussen, Häbler, *et al.* (1926). On the other hand, in inflammation there enters a significant increase of the osmotic pressure of the tissue fluid, and these workers maintain that the osmotic (and oncotic) hypertonicity of the tissues is the chief factor operative in inflammatory exudation and swelling. This again is in agreement with the findings of Hoff and Leuwer (1926) that the passage of Congo red through the capillary wall is dependent on osmotic pressure and the existing ionic conditions.

c. Calor. The heat of inflammation is largely a function of the increased amount of blood brought to the affected region. In addition it seems certain that the accelerated chemical activity of the inflammatory zone produces a significant local heating which is a contributory factor.

d. Dolor. Several physico-chemical factors, in addition to any direct mechanical stimulation of sensory nerves, coöperate to produce the pain of inflammation. One such factor is a departure from isotonicity. The injection of indifferent isotonic solutions is painless, but the injection of solutions of other strengths ($\Delta = -0.34$ to -1.30) evokes momentary pain before buffering takes effect in the same progressive degree as the departure from isotonicity (Ritter, 1905; Braun, 1914; Rhode, 1921). That the tissue fluids maintain an altered osmotic pressure during inflammation is well established (p. 364).

The disturbance of the H—OH balance is a second factor of fundamental importance with relation to pain. The experiments of v. Gaza and Brandi (1926a, 1927) showing a direct production of pain by the injection of acid and the relief of pain in patients by alkaline injections have been described in an earlier paragraph (p. 371).

A third factor is a disturbance of the sodium-calcium-potassium balance so that an excess of potassium appears in the exudate. This latter ion has been proved to be preëminent among the neutral salts in eliciting pain. For example, the injection of isotonic potassium chloride solution is highly painful, as Bommer (1925), Hoff and Leuwer (1926) and Häbler and Hummel (1928) have all proved. This type of experimental pain lasts up to one hour after injection. Since there is an increase of potassium released from the cell during acute inflammation

(Schade, Claussen, Häbler, *et al.*, 1926; Tutkewitsch, 1927), it is reasonable to conclude that this factor plays a part in the causation of pain. The potassium content of pus and exudate increases with the severity of inflammation, and in purulent exudates may reach 51 to 205 mgm. per cent (Häbler, 1929). On the other hand, the conditions prevailing in cold abscesses are also significant from the standpoint of pain. As Ritter (1905) and Schade, Claussen, Häbler, *et al.* (1926) have found, there is little or no alteration of the osmotic pressure in this form of inflammation; moreover, the acidity is slight and the potassium content is not increased. In correlation with these characteristics it is well known that pain is usually not a concomitant of the cold abscess.

Finally, the pressure of turgescence within the tissues, which represents the sum total of the various forces at work there, is operative. This has to do especially with the phenomenon of pulsating inflammatory pain which Schade has shown is brought to subjective consciousness when external pressure upon the blood vessels is made to equal the internal pressure.

e. Functio laesa. The disturbance of the several physico-chemical constants is responsible for the reduced functioning of the tissues affected. The osmotic hypertonicity is inimical to cell function. Cell volume, including nuclear volume, is thereby reduced (Hamburger, 1904), while phagocytosis is decreased (Hamburger and Hekma, 1907). The acidity of inflammatory tissues is sufficient to produce a cloudy swelling of protoplasm (Hamburger, 1904; M. H. Fischer, 1911) and apparently fatty degeneration as well (Fischer and Hooker, 1916). The normal enzymic processes operate at an optimum pH value, alterations in which bring about a reduction of activity which may reach complete cessation. As an illustration of the dependence of function on proper hydrogen-ion concentration may be mentioned the experiments of Rona and Neukirch (1912) who found that the optimum peristalsis of the rabbit's intestine occurred at pH 7.35 (practically isoionic with blood), whereas departures from this level led to sluggishness and at pH 5.7 peristalsis ceased. These disturbances in the several physico-chemical constants also bring about fundamental alterations in the tissue colloids. The normal swelling effect of some of the anions and kations is reversed in infected wounds (v. Gaza and Wessel, 1923), although this may not be true of aseptic types (Tammann, 1933). Such a partial reversal is apparently related to the altered conditions of acidity (Schade).

V. Factors influencing healing. *1. Age.* It is well known that age has a potent influence on the rate of healing. This relation has been investigated with great precision by du Noüy (1932) who has shown that the age of a person can be expressed in terms of a constant which in a formula of the healing rate corresponds to his physiological activity. Or, stated differently, the index of cicatrization of a wound indicates exactly the age of the patient.

The increased rapidity of healing which is characteristic of younger individuals is usually accepted as being due to an increased rate of cellular proliferation. Another interpretation, however, is that this may be the result of a diminution in the amount of retardation entering into the process (Harvey, 1929). In fact, Howes and Harvey (1932) interpret their experiments to indicate that healing in the young is more rapid than in adults because fibroplasia begins earlier and is less retarded, and not because the rate of fibroplasia is greater.

2. Mechanical Pressure. The favorable influence of selective, purposeful mechanical pressure on wound healing has been discussed by Blair (1924) who lists the four basic ends to be gained as: elimination of dead spaces; control of oozing; limitation of stasis; limitation of the amount of plastic substance that pours into the wound. Of more fundamental interest is the demonstration by Twyman (1922) that epithelium proliferates readily when subjected to pressure that restrains the growth of connective tissue. Hence by regulating the bandage pressure to an amount which corresponds to that required to blanch the finger nail, the granulation capillaries are compressed or collapsed and the tissue is repressed, while epithelial growth proceeds unaffected.

3. Temperature. Ebeling (1922a) has determined the effect of temperature on the rate of cicatrization in the alligator. For a rise of 10°C. the rate increased about two-fold. This result is to be expected since wound healing is related closely to growth and regeneration. It is a fundamental fact that temperature changes affect the metabolism and development of certain organisms in the same manner that they do many chemical reactions. In the latter instance the existence of a temperature coefficient of approximately 2.0 is well known. In spite of the complexity of the factors which bring about cicatrization, Ebeling concludes that the velocity of the reparative phenomena depends on the rate at which certain chemical changes take place. The rôle of temperature doubtless is a factor in the favorable results produced by sympathectomy in mammals, since a definite increase in the warmth

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of the affected part is a characteristic of the resultant hyperemia. On the ear of the rabbit this elevation has been found to be some 5°C. (Rieder, 1924).

The application of thermal radiation to wound healing was found by Fuke (1932) to reduce appreciably the period of repair in rats.

4. Electricity. The influence of high frequency electric currents (25,000 volts and 2–3 milliamperes produced by a MacIntyre tube) has been recorded by Massimi (1921). Each day the sterile tube was placed for 5 to 10 minutes in experimental wounds on animals. A hastening of wound healing was observed which Massimi attributes to the promotion of sterilization and hyperemia.

Diemer (1927) believes that the utilization of a constant galvanic current acts through electrolysis and the liberation of endosmotic processes to bring about a local concentration of nutritive substances which can be utilized by the cells in their general metabolism.

5. Radiant Energy. a. Thermal rays. Fuke (1932) tested the effect of heat rays (passed from a "solar" lamp through glass painted with soot) on wounds in white rats. Healing was reduced from 14 to 11 days.

b. Infrared rays. No significant benefit from this type of radiation was obtained by Fuke (1932) on rats, but Lemairiée (1931) believes that the healing of small human wounds, in particular, is hastened thereby.

c. Phosphorescent light. The cold rays of phosphorescent light were utilized by Schwartz (1933) through the application of a salve or plaster. Such continuous administration was said to accelerate wound healing in guinea pigs and rabbits through a quickening of epithelial regeneration.

d. Ultraviolet light. Torraca (1921) tested the influence of heliotherapy at an altitude of 2900 meters on wounds of guinea pigs. Some animals were exposed to sunlight 10 to 11 hours daily. Others were kept in the shade at the same temperature. The first group showed a considerably faster healing rate which Torraca attributes to the direct action of radiation since the wounds were aseptic and an indirect bactericidal action could be eliminated.

Somewhat similar experiments, also on guinea pigs, were performed by Gianotti (1927). This worker found that animals with bandaged wounds experienced the same healing rate in the sun as those held completely in the shade. Animals exposed freely to the sun healed their wounds 35 per cent faster than either of the first two groups,

while immobilized animals with the wound alone receiving solar light responded even better (64 per cent). The action of the sun's rays was held to be essentially local.

Various workers have tried the effect of nonsolar ultraviolet light on wounds. Only Markuse (1933) concludes that the healing time is not hastened by such radiation, yet even he found some benefit in old rats and rabbits. On the contrary, an accelerating influence has been recorded by Byčenkow and Kruglov (1929) on the rabbit, Lemairiée (1931) on man, Fuke (1932) on rats, Pollaczek (1933) on man and the guinea pig, and Repetto (1934) on rabbits. Treatment in excess of mild dosage reverses the effect so that it becomes definitely injurious (Pollaczek, 1933).

e. Roentgen rays. That the recorded experiences with relation to irradiated wounds are somewhat conflicting is not astonishing in view of the different experimental conditions under which the several tests have been conducted. A negative or retarding influence has been reported by Haberland (1923) and Podestá (1930) on the rabbit, and by Pohle, Ritchie and Wright (1931) and Pohle and Ritchie (1933) on the rat. Opposed are the findings of Schürck and Tsudi (1929), Fukase (1930) and Buhtz (1933) on the rabbit, Businco and Cardia (1930) and Nathanson (1934) (with the gamma ray of radium) on the dog, and D'Istria and Di Bella (1930), all of whom found a beneficial effect.

Ritchie (1933) concludes from a review of the literature that, in spite of conflicting evidence, it appears that small doses stimulate the healing process. On the contrary, he believes that when larger doses, approximating those used in the treatment of malignant tumors, are administered within 48 hours of the incision there is a definite retardation. The retarding effect is less on epithelium than on connective tissue (Pohle and Ritchie, 1933). This interpretation is in accord with the conclusion reached by D'Istria and Di Bella (1930). Nathanson (1934), utilizing the gamma ray of radium, also agrees that small and medium doses applied immediately after an incision induce an accelerated healing, whereas retardation follows the use of larger doses. Moreover, a retardation was found in all wounds exposed to any dose 24 hours after the incision, the degree of slowing varying directly with the dosage. In addition, wounds subjected to varying doses 48 hours after the incision were retarded only by the larger doses whereas no change followed smaller ones.

6. Metabolism. Clark (1919), was the first to study the effect of

diet on the healing course. The sole influence observed was upon the latent period, whereas the periods of contraction and epithelization remained unchanged. Hence the type of diet used influenced the total period of healing proportionately as it affected the latent period. High protein diet eliminated the latent period completely, whereas high fat diet prolonged it to six days. High carbohydrate and mixed diets were intermediate in effect (four and three days, respectively). The fact that proteins stimulate cellular proliferation while fat acts as an inhibitor has been demonstrated in various other ways (Smith and Moïse, 1924; Carrel and Baker, 1926; Osborne and Mendel, 1926).

Somewhat different are the results of Harvey and Howes (1930) who studied the progress of wound healing from another standpoint (*i.e.*, by testing the tensile strength of the regenerating connective tissue). In this instance the duration of the latent period was not affected, but once growth was started the velocity of fibroblastic repair increased distinctly under high protein diet. As a result, the maximum strength of the wound was attained considerably earlier than with animals on standard diet. Of interest in this regard are the observations of Doberauer, Hoke and Pittrof (1916) that the fattening of rabbits with oats ("Trockenfütterung") or the injection of gelatine leads to an intensive new formation of connective tissue in primary healing wounds in comparison to the results obtained from a diet of green fat.

Consistent with such results is also the clinical experience of Herrmannsdorfer (1927) and others, confirmed by Reimers and Winkler (1933) on dogs, that acid diet (high in fat, proteins and acid phosphates) influences favorably the cleanness and healing rate of wounds. Reference only can be made in this place to the numerous relations that have been established between local acidity and the phenomena of wound healing (p. 367 *et seq.*). An attempt to elucidate the influence of metabolism on the reactive processes of wound healing has been made by Leupold (1928), but his results are far from final since the unleashing of tissue new-growth is due to manifold interacting physical and chemical processes.

Some things are known pertaining to the effect of inadequate diet and starvation on wound healing. Morgan (1906) demonstrated long ago that amputated legs of starved salamanders regenerate in the absence of an external source of food as rapidly as do those of well-fed animals. Similarly, Howes, Briggs, Shea and Harvey (1933) found that the rate of return of healing strength in stomach wounds was not appreciably affected in adult rats by complete starvation or by half-

adequate diet over a short time, whereas there was a considerable retardation in young rats on half-adequate diet. In general, it is well known that diseases that affect the general metabolism also are unfavorable to wound healing. In particular might be mentioned the retarded healing and tendency toward infection experienced in diabetics and in malnourished infants.

7. Vitamins. It has frequently been stated that the vitamins in general exert an influence on regenerative processes, but until recent years no exact experimental information has been available on which definite opinions might be based.

a. General avitaminosis. Ishido (1923) fed guinea pigs and rats on a vitamin-free diet and reported that they showed a plain tendency toward delayed wound healing in comparison both to normally nourished animals and to those underfed on a normal diet. In the individuals suffering from vitamin deficiency the adherence of the walls of the wound is faulty and there is a tendency toward an improper new formation of connective tissue. In the latter instance fluid collects in the floor of the wound, while cavity formation, necroses and susceptibility to infection are additional characteristics. One wound did heal in such an animal but it required twice as long as the normal cicatrization period.

These results are in accord with similar experiments carried out by Lauber (1934) on the rat and guinea pig. The latter animal was used for C-avitaminosis alone, since this condition cannot be produced in rats. Animals subjected to vitamin-free diet showed a delayed healing. Lauber suggests that, as for hormones (1933), the favorable effect of vitamins is accomplished through altered cellular metabolism. Moreover, both groups are considered to belong in the same biological category; their respective representatives have both synergistic and antagonistic relations to each other.

b. Vitamin A. Roegholt (1929) did not find that A-avitaminosis changed the rate of healing in epithelial wounds. It seems, however, that his experiments cannot be considered crucial since insufficient time was allowed to test the full effect of avitaminosis.

The importance of the time element is brought out in the experiments of Saitta (1930a) who first examined the course of healing in animals that had been on a diet free of vitamin A for 66 days. No significant differences were found in the healing rate over controls, even when the former were painted locally with the vitamin A product. In another series, 105 days on the diet, untreated wounds remained unhealed

until they became infected or death supervened. On the other hand, wounds painted with the vitamin extract healed in 13 days without complications in comparison to a corresponding period of 16 to 18 days for the controls.

Lauber (1934) conducted what is doubtless the most careful investigation yet performed on the relation of vitamins to wound healing. As far as is possible under the present state of knowledge he used standardized, controlled dosages. In a preliminary series of experiments on the effect of vitamin A, Lauber established that pretreatment of rats with this vitamin was without effect on the healing rate of wounds subsequently produced. A second series consisted in the daily, oral administration of vitamin A following the infliction of skin wounds. He found that moderately strong dosage (four rat-units per diem) led to a remarkable acceleration, some lesions healing in 9 days in comparison to 18 to 25 days for the controls. Over-dosage to the amount of 200 units produced no departure from the normal time, whereas 40,000 units strongly inhibited healing (36–52 days).

The favorable effect of oils or salves containing vitamin A applied directly to human traumatic wounds has been described by Horn and Sándor (1934). Both epithelization and granulation formation were speeded, and, through the more rapid growth of the latter, secondary infections were restrained.

c. Vitamin B. Saitta (1930c) reported that B-avitaminosis diminishes greatly the power of cicatrization in wounds of the skin, subcutaneous layer and muscle of pigeons and rats, whereas oral administration of an extract of cereals was found to nullify the dangerous effects of avitaminosis. On the other hand, he treated the wounds directly with concentrated vitamin extract and records that cellular repair was activated so that healing occurred sooner than normal.

Somewhat more negative in nature are the findings of Lauber (1934) who emphasizes the unreliability of results obtained from experimentation with the B-complex, due to the lack of standardization as to the exact amounts of this vitamin received in a B-rich diet. Although he holds his results to be tentative, awaiting a satisfactory standardization, he could find no difference between the healing time in animals receiving yeast in their diet over controls fed on an ordinary ration.

Still more directly opposed to the conclusions of Saitta are the experiments of Larcher (1927) who found that in pigeons with B-avitaminosis there was an equal, or even faster regeneration of traumatic injuries

to the muscle or nerves than in control animals. This was notable in the nerves where it occurred in spite of the induced polyneuritis.

d. Vitamin C. Saitta (1929) fed guinea pigs on a scorbutic diet and accomplished C-avitaminosis in 15 days. As the scorbutic effect increased, the process of cicatrization became less active and was finally arrested. Conversely, Saitta found that the direct application of an extract of vitamin C to wounds facilitated the process of healing.

Lauber (1934) also used guinea pigs, since rats apparently synthesize this vitamin. Animals on a diet rich in vitamin C did not differ from controls in their healing rate, yet, as in the experiments with vitamin B, such unstandardized results are held to be only provisional.

e. Vitamin D. Saitta (1930b) has reported his experience with vitamin D gained through the use of irradiated ergosterol. He administered ergosterol orally, by direct painting on the wound, and by a combination of these methods. In one series of experiments the results were unfavorable. Direct application resulted in nearly doubling the healing time. Oral administration gave still worse results; at 20 days the animals were emaciated and in bad condition, whereupon the treatment was suspended; but even after 44 days the wounds were still open for half their lengths. The exaggerated dosage used in this series was held responsible for the production of the toxic effects which have been recognized by others. In a second series, using another ergosterol product, the normal healing time was equalled both after oral and direct application, while a combination of the two methods led to a slight delay.

Delayed wound healing, induced in rabbits as the result of overdosing with vitamin D, has been described by Tammann (1931b). Necrosis and calcification also occur in the arterioles of the deeper granulation tissue.

Lauber (1934) also has experimented with this vitamin. Foretreatment or concurrent treatment with varying dosages (including strong overdosage) failed to produce any effect on the healing time in the rat. Lauber refers to the work of von Berberich and Fischl who found the vascular injuries due to vitamin D to be manifested soonest in the cat (3 weeks), later (3–4 months) in the rabbit, and still later in the rat. Such individual variability he believes may easily explain the differences between his negative results and the positive ones of Tammann. Lauber also emphasizes that all the investigations conducted on lower mammals do not yet afford safe conclusions which can be transferred directly to human therapy.

8. Hormones. Among the endogenous agents ("Körpereigene Reize" of Bier, 1917a) having an effect on the course of tissue repair are the hormones. That our present state of knowledge concerning the action of these substances is somewhat contradictory and lacks finality is due to several causes. Absence of standardization in the products administered, variability in the dosage, and wide differences in the way the experiments have been conducted all conspire toward confusion and contradiction.

Bier (1917a) has discussed the probable rôle of hormones in tissue regeneration, and Lorin-Epstein (1927) and especially Fränkel (1928–1932) have followed his train of thought. Fränkel, in particular, experimenting with bilateral fractures, believes to have identified two kinds of hormone action; one is a "plastic hormone" which sensitizes the regenerate for the second or "differentiation hormone." Whether these are specific hormones or are special manifestations of hormones already known is not proved.

But the history of the relation of hormones to repair is somewhat older than this. Aievoli (1890) was the first to establish a relation between cicatrization and local hormonal influences. Testis parenchyma laid on wounds was found by this pioneer investigator to result in earlier healing. Many years later Voronoff and Bostwick (1918) and Aievoli (1923) extended these experiments to certain other endocrine organs. Pancreas, suprarenal, thryoid and testis in the form of pulp or slices were applied directly against the wound. Under these conditions epithelization and granulation formation were delayed by the pancreatic tissue and expedited by the suprarenal and thyroid, while the strongest healing influence was afforded by the testis. From such beginnings a considerable body of information has developed, much of it produced by Italian investigators. Some of the accounts are brief and doubtless reflect a superficial and uncritical approach. It will be simplest to consider separately the experimental and clinical findings recorded for each of the major endocrine organs.

a. Gonads. The pioneer experiments of Aievoli (1890) and Voronoff and Bostwick (1918) have been alluded to in a preceding paragraph. In 1923 Aievoli returned to a consideration of hormonal influence and presented his collective results on animals and man. His earlier conclusion as to the favorable influence of heterologous testis material applied as slices or reduced to a pulpy mass was reaffirmed for human wounds. A purely local action for this type of experiment was held, since more remote, untreated wounds on the same individual were not

influenced. A later communication (1931) argues against the contention of Marino (1931) who believed that the accelerating effect of testis tissue is not due to a thermostabile hormone but to a thermolabile trephone. Aievoli here presents evidence in support of his claim for a hormonal action, and in this he is supported by Gussio and Gibilisco (1931) and by Ciantini (1933).

Favorable results from the direct application of sliced or pulped testis to wounds have also been recorded by Siciliani (1927), Marino (1931), Gentile (1931) and Gussio and Gibilisco (1931), some of these results being obtained on clinical patients as well as on experimental animals. Unlike other investigators, Gussio and Gibilisco (1931) report a delaying action on the healing of wounds in the dog when heterologous testis (beef) was used, although they found an acceleration after homologous gland therapy.

The administration of testis extract has also produced favorable results. Application by painting or salving the wound surface, by subcutaneous injection or by oral administration have been employed. Dal Collo (1926), Mumoli (1927), Pozzuoli (1927), Chiasserini and Ferretti (1927), Ciambellotti (1929), Ceccarelli (1930), Ciantini (1933) and Lauber (1933) all obtained a significant shortening of the healing time by one or more of these methods. Parenteral administration of testis extract was found ineffective only by Mumoli (1927) and Gussio and Gibilisco (1931). Lauber (1930) records a favorable result with ovarian extract and Dal Collo (1926) even reports positive results of ovary therapy on male guinea pigs and testis therapy on females. The investigation conducted by Lauber (1933) is probably the most carefully performed and most trustworthy of all. He found the male hormone effective in both sexes, but especially in the male. The female hormone had a marked influence on hastening healing in the female, while it had little or no effect on the male.

Surgical removal of one or both ovaries or testes prior to the infliction of experimental wounds on rabbits and guinea pigs has no demonstrable effect on the course of wound healing (Merlini, 1928; di Natale and Midana, 1931). On the other hand, Merlini claims that the previous intraperitoneal implantation of one or two testes in rabbits hastens the healing plainly, whereas the corresponding experiment with ovarian implants is without influence.

b. Thyroid. Voronoff and Bostwick (1918) and Aievoli (1923) agree that sliced or pulped thyroid gland applied to the wound surface promotes quicker healing. In a similar manner the favorable influence

of thyroid extract administered variously has been recorded by Siegmund (1910), Ceccarelli (1930), Eppinger (1931), Vitale (1933), Ciantini (1933), Lauber (1930, 1933), and Kosdoba (1934a). The researches of the latter two investigators are especially meritorious. Lauber found the time of healing was reduced nearly 50 per cent.

Partial (or total) ablation of the thyroid has been performed by Merlini (1928), Natale and Midana (1931), Ciantini (1933), and Kosdoba (1934a). All agree that this results in a definite retardation of healing. The converse experiment of implantation of the thyroid gland was without influence in the experience of Merlini (1928), whereas Kosdoba (1934a), whose experiments are perhaps more trustworthy, found a resulting acceleration of healing. In general, Kosdoba attributes the chief efficacy of the thyroid hormone to an influence on the connective tissue and blood vascular system, while at the same time he believes the biochemistry of the wound contents is affected.

c. Parathyroid. Melina (1933) reported that the glandular extract applied locally promotes early repair, whereas parathyroidectomy retards the process of cicatrization. In complete disagreement are the results of Lauber (1933) who observed a retardation in the later stages of healing following the injection of therapeutic doses of parathyroid hormone.

d. Pancreas. Voronoff and Bostwick (1918) and Aievoli (1923) concluded that the direct application of pancreas tissue to wounds delays and even stops the healing course. Contrariwise, Chiasserini and Ferretti (1927), Clairmont (1928) and Ceccarelli (1930) agree that insulin applied as a salve or painted on the wound had an accelerating effect. On the other hand, Lauber (1933) observed no difference in the healing time of animals treated with insulin in comparison to controls, whereas the vasodepressor-principle preparation had a marked accelerating action. The favorable influence of insulin on atonic wounds and ulcers has been commented on clinically, as has the use of vasodepressor preparations on trophic ulcers and especially those of Raynaud's disease.

e. Suprarenal gland. Voronoff and Bostwick (1918) and Aievoli (1923) agree that the direct application of suprarenal tissue to wounds induces the growth of rich granulations, while Lauber (1933) also concluded that adrenalin injection shortens the course of healing strikingly. Conversely, Santorsola (1931) found after the removal of one gland that trophic disturbances resulted, together with a diminution in the power of tissues to react and regenerate. As a result, the healing

rate for aseptic ulcers in rabbits was slowed progressively in proportion to the elapsed postoperative time.

In direct opposition to these several agreements are the extensive experiments of Kosdoba (1934b) who studied the effect on healing time of a hormone increase produced by various means (*viz.*, nicotine; physostigmine; electrical stimulation; adrenalin given internally and subcutaneously; homotransplants). In every type of experiment an attempt to increase the amount of hormone in the animal resulted in a lengthening of the healing period. The opposite type of experiment, in which the suprarenal was removed or destroyed, resulted in some of the wounds equalling the rate of healing in control animals.

f. Hypophysis. Ceccarelli (1930) concluded that the direct application of an extract of the anterior lobe of the hypophysis accelerates healing somewhat in the first week but does not shorten the total healing period. On the contrary, Lauber (1933) obtained rapid healing in animals injected both with "Hypophysin" and with "Prolan." The slowest individuals to heal under Prolan treatment were almost always males.

g. Miscellaneous. Voronoff and Bostwick (1918) and Aievoli (1923) obtained negative results following the direct application of spleen tissue to healing wounds. Brezovsky (1930) used a salve ("Granormon," containing extracts of thymus, testis, ovary and thyroid, besides the vitamin of Ol. Jecoris Aselli) on human wounds and found a considerable acceleration of healing. Convinced of the effect of the "thymus hormone" as a stimulator of the regenerative capacity of tissues, he (doubtless mistakenly) attributes the beneficial results to the thymus component. Equally remarkable is the report of Michaïl and Vancea (1930) on the accelerating effect of an extract of the lacrimal gland. McJunkin and Matsui (1931) have described a specific growth-promoting substance in homologous epidermis which when applied to wounds stimulates epidermal regeneration.

9. Nervous Influences. As early as 1890 Morpurgo advanced the claim that the sympathetic nervous system exerts an inhibiting effect on the reparation of tissues. This conclusion was supported by the observation that when the appropriate nerves are cut the restoration is more rapid than normal.

Nevertheless, to Liek (1902) belongs the credit for the first thorough, modern investigation on this subject. In fact, later workers have only confirmed and extended slightly his fundamental conclusions. Liek resected the sympathetics or removed the superior cervical ganglion

unilaterally in rabbits and observed that the ear on the operated side became hyperemic. Equal patches of skin, between 0.25 and 1.0 sq. cm. in area, were then removed and the progress of healing watched. The wounds on the operated side healed quicker through a more rapid growth of granulations and epidermis. Regeneration paralleled the degree of hyperemia. The control side, on the other hand, retained a torpid appearance for a longer period, became clean later, and took double the time for completing healing. Liek concluded that the favorable results are due to sympathectomy, and, more precisely, to the hyperemia produced by it. Essentially corroborative findings have been reported by Leriche and Haour (1921), Rieder (1924), Placintianu (1924), Locatelli (1926), Fontaine and Jung (1928), and Sozon-Jaroševič and Sewald (1932) on rabbits, and by Rieder (1924) on dogs. The latter found even better results were obtainable through stimulation of the vasodilator mechanism, while the best effect was gained by this treatment combined with sympathectomy. Another type of attack to demonstrate the influence of the sympathetics was made by Sozon-Jaroševič and Sewald (1932) who hoped to stimulate ganglionic activity by the injection of sterilized pus. Under these conditions wound healing was greatly delayed.

In 1917 Leriche and Heitz stated that the effects of periarterial sympathectomy in human patients were (after a relatively brief, primary contraction of the artery) vasodilatation, increased temperature, a rise in arterial pressure, and a stronger pulse. These statements were confirmed by Papilian and Cruceanu (1924). Later, Leriche (1920, 1921) and Leriche and Fontaine (1928) reported on the effect of periarterial sympathectomy on the healing of human wounds. The denervation was accomplished by stripping away the adventitia for a short distance. The resultant accelerating effect on cicatrization was especially striking in obstinate ulcers and atonic wounds, epithelization being achieved in as short a period as eight days. Essentially similar were the results of Papilian and Cruceanu (1924) on experimental wounds made on the ear and leg of dogs. Schönbauer and Whitaker (1925) also have recorded that not only did wounds on the leg of the dog heal faster after periarterial sympathectomy but also that deep wounds of the stomach lining healed with rapidity following similar operative treatment. On the other hand, Rieder (1924) was unable to establish more than a very limited and scarcely detectable advantage in the healing of wounds in the dog following periarterial sympathectomy, but did find that this procedure was followed by more or less prompt healing in cases of trophic ulcer

in man. However, Rieder's account should be consulted for a critique of the Leriche type of operation and its exact relation to healing.

Schönbauer and Whitaker (1925) experimented with the effect of periarterial sympathectomy on wounds of the dog's leg intentionally infected with introduced bacteria. They report that the wounds cleared and healed rapidly. On the contrary, Rieder (1927) could find no significant acceleration in the healing time under these conditions, although resection of the rami communicantes or of the sympathetic trunk did lead to markedly earlier repair.

10. Reparative Stimuli. The rôles of tissue hunger, products of cell destruction "wound hormones," trephones, mitogenetic rays, etc., have been discussed in an earlier section (p. 333 *et seq.*).

11. Irritants. An evaluation of the effect of some of the substances which have been applied directly to the wound surface is not always easy because of the possible involvement of an irritative stimulus. That irritants are a real factor is suggested by Carrel's (1921) experiences in shortening healing through the local application of such substances as turpentine. In fact, the healing rate of wounds has even been stimulated by injecting distilled water into experimental animals (Ruth, 1911).

12. Infection and Disease. Chief among the factors that delay healing is infection. It is impossible to enter here into any discussion of this topic, although some reference to its relation to the regenerative process has already been made. Carrel (1924a) and Kiaer (1927) have even recorded delay in healing due to the existence of a distant abscess elsewhere in the body. The inhibiting effect of syphilis has been discussed (Milian, 1919; Darnell, 1920), and reports of the influence of other systemic diseases and changes are numerous.

VI. THE NATURE OF THE FORMATIVE STIMULUS. Many biologists have speculated on the way in which tissue loss leads to regeneration (*cf.* Marchand, 1924). Virchow (1858) taught that inflammatory growth resulted from external stimuli, or inflammatory irritants, which incite the cells to grow and multiply, but Weigert (1896) denied this power to any external agent. Also many years ago, Welch (1891) presented the hypothesis that the formative stimulus of Virchow consists of substances acting from without which directly stimulate cells to growth and proliferation; the stimulating principle would be, therefore, chemical in nature.

1. Theory of Carrel. In the absence of any pertinent evidence one could assume that the formative stimulus might be either something

that removes an obstacle to growth or something that directly promotes growth. Carrel studied this matter and came to the conclusion that the resting condition of adult cells is not due to a restraint which prevents them from displaying activity. Hence he set about examining the substances in blood and other tissues of animals of all ages with the end in view of finding the agent which directly promotes growth. After a time it was discovered that embryonic tissue-juices bring about an intense proliferation of epithelial cells and fibroblasts (Carrel, 1912), so that through this property pure strains of tissue cells can be maintained indefinitely *in vitro* (Ebeling, 1922b). This quality of embryonic tissue does not depend upon its component amino acids. On the contrary, the active principle is a protein which is easily caused to lose its activity, wholly or partially, by such means as shaking or by heating at 56°C. Many other proteins proved to be inactive, but the higher cleavage products of certain proteins were as active stimulating agents as are the embryonic proteins (Carrel and Baker, 1926). The incomplete digests of ox fibrin, casein, egg albumin, liver, testis, thymus, hypophysis, etc., increased the rate of growth, but the volume of new tissue differed according to the nature of the protein and the degree of its digestion. Additional hydrolysis of these proteins yielded nutrient substances, but all activity was lost when the solution consisted exclusively of amino acids. It was concluded that the ability of tissue-juice to promote growth should be attributed to the ease with which its proteins can be transformed into peptides by tissue enzymes. Baker (1929) then studied the effect on the growth of fibroblasts of glutathione and hemoglobin when added to casein digest, glycocoll and nucleic acid, and showed that both glutathione and an iron compound are necessary for the life of fibroblasts *in vitro*. These results have been confirmed and extended by Hammett (1929) and Hammett and Reimann (1929) who conclude from experiments on plant and animal tissues that the sulphydryl radical is the essential stimulating agent in cell multiplication.

We are thus in possession of a mass of experimental evidence which shows that the rôle of formative stimulus is played by embryonic proteins and split-products from certain proteins, and that the varied effects of these compounds depend on the presence or absence of certain molecular groups. Under normal conditions, these substances are not contained in blood plasma. But they can be found in leukocytes, thyroid gland, bone marrow, kidney, ovary, muscle, etc., of the adult animal. It is probable that, *in vivo*, any lesion of a tissue produces the diffusion in the lymph of intracellular substances. At the same time, growth-activating polypeptides may be manufactured by the leukocytic ferments from cell débris and coagulated fibrin lying on the surfaces of the wound (Carrel, 1930).

In addition to their function in combating infection, leucocytes doubtless have other fundamental activities in the nutrition of the organism and in wound healing. Carrel's (1924b) studies have shown that among their peculiar properties is that of their food requirements which differ markedly from those of fixed cells:

> They multiply actively when fed upon blood plasma, red blood corpuscles, muscle tissue, or protein precipitates. On the other hand, they are generally killed by embryonic proteins and protein split-products at a concentration which is optimum for the multiplication of epithelial cells and fibroblasts. They set free in their medium growth-promoting substances, which consist of embryonic proteins, or of protein split-products. They act simultaneously as food transformers and nurses. The presence of many leukocytes on the granulating surface of a wound, and the infiltration by them of the fibrin deposits render plausible the hypothesis that they play the part of a formative stimulus (Carrel, 1930).[5]

The next point discussed by Carrel (1930) covers the causal relations existing between the loss of a fragment of tissue and its regeneration. How can a traumatic injury bring about the repair of the wound for which it is responsible?

> In superficial epithelial wounds, the initiation of healing seems to be due, not to leukocytic secretions, but to substances set free by the traumatized cells themselves. Through two oblique incisions, Fischer resected a triangular segment of a slowly growing colony of a pure strain of cells. Then, the whole colony started to grow more actively, and healing took place. During the process, a drop of Tyrode solution was placed on the little wound for varying periods of time. The testing of this drop showed that the wound had manufactured thrombokinase and also growth-promoting substances. To these substances must be due the increase in the growth of the colony following the incisions, and the rapid healing of the wound. Healing, then, seems to result not from a direct stimulation of the cells by the traumatism, as Virchow thought, but from substances liberated by tissues injured by this traumatism.
>
> Two identical wounds were made on the skin of dogs. After a minute cleansing of its surface, the experimental wound was dressed with dead subcutaneous connective tissue. Both wounds were covered with gauze pads. After more than three weeks, no beginning of cicatrization was detected on the experimental wound, while the control wound had cicatrized in a normal manner. Healing is not initiated by the traumatism or the loss of tissues, but by substances resulting directly or indirectly from the traumatism. The hypothesis was further investigated in a study of the effect of mild irritants, such as turpentine, embryonic tissue pulp, and staphylococci, on the duration of the latent period. In every

[5] With these conclusions as to the rôle of the leucocyte as the active stimulating agent for wound growth Burrows (1924) is in disagreement. This worker holds that they inhibit growth of the connective-tissue cells in cultures when they occur in larger numbers, and protests that he has seen the most active growth always in wounds containing but few leucocytes.

case, cicatrization started in less than two or three days. This indicated that the shortening of the latent period was not due to the irritant itself, but to some product of the irritation of the tissues, and possibly to the secretions of the leukocytes, which are present in great numbers in an irritated wound. Obviously, the traumatism determines directly or indirectly the production by the tissues themselves of the formative stimulus.

A third consideration treated by Carrel (1930) pertains to the rôle of the blood plasma. Its relation to the healing process must be of prime importance since through it the composition of the pericellular fluid is regulated. However, blood plasma not only does not contain any substance that promotes the growth of epithelium and fibroblasts, but it actually possesses the important property of inhibiting cell proliferation (Carrel and Ebeling, 1923).

This inhibiting effect increases considerably with age. The curve expressing the increase in the inhibiting effect of serum in function of age inclines downward very rapidly during the first months of life, and very much more slowly in old age. The amount of tissue produced by a pure culture of fibroblasts in blood plasma can be used for measuring the age of an animal. It is, in fact, the only method which we possess for measuring *in vitro* the progress of time on the organism. The growth-inhibiting effect of serum must be attributed not to amino acids or salts, but to lipoids which it contains. Lipoids extracted from chicken brains, chicken liver, chicken eggs, and embryonic tissues have likewise an inhibiting action. The lipoid constituents of the blood become more inhibiting with age. As cell multiplication depends on the hydrolysis of proteins, it is plausible that the serum restrains growth on account of the anti-enzymatic effect of its lipoids.

We may expect a similar effect of plasma on tissues *in vivo*. It is well known that age impresses its stamp on the rate of healing. This subject was investigated with great precision during the War by du Noüy, who discovered a mathematical relation between the area of a wound, the age of the patient, and the index of cicatrization. This relation is so constant that, with the help of du Noüy's table, the index of cicatrization of a wound exactly indicates the age of the patient. This phenomenon seems to be due to the chemical changes occurring in the composition of serum when the age of the animal advances. More evidence has been produced by a study of the effect on the rate of healing of a pathological modification of the blood. . . . These experiments demonstrated that a modification in the composition of blood plasma brings about a modification in the rate of healing, and that the growth energy of the tissue cells of a wound is inhibited by plasma in the same manner as that of a pure culture in a flask. It is probable that blood serum also possesses the important function of determining the differentiation of the cicatrizing tissue which takes place when healing approaches completion. Such a phenomenon occurs in tissue cultures when actively growing cells are treated with heparined plasma or serum. The rate of growth decreases and differentiation products appear. . . .

This mass of observations and experiments proves that blood plasma has an effect on fixed cells opposed to that of embryonic proteins and protein split-

products. While these substances produce cell multiplication and dedifferentiation, plasma has a tendency to inhibit proliferation and bring about differentiation, probably through the lipoids and proteins which it contains. The variations in the rate of wound healing produced by age and diet are very likely dependent upon these same substances. Finally, blood plasma, on the completion of the process, restores the tissues to the resting condition of normal life.

2. Theory of Burrows. It has been shown (Morgan, 1906) that the growth in regenerating tissue is not due to food and oxygen alone. The legs of starving salamanders regenerate as rapidly as those of well-fed animals. The difference between the two is that the starving salamander suffers an excessive atrophy of all its organs; yet the regenerating tissues derive their food supply directly from the differentiated cells less favorably located in the body. Through the use of tissue cultures Burrows (1924, *et ante*) has attempted to discover the significance of these facts and the nature of the conditions or stimuli, other than food and oxygen, which are necessary for cell growth. In cultures of embryonal cells, adequately supplied with food and oxygen, he observed that these cells are unable to grow except when so crowded that some product of their normal oxidation cannot escape. Thus it was found that growth can be stopped by washing the cells with serum or by placing them in a relatively large amount of medium which in some way keeps the concentration of this oxidation product below a certain level. The concentration of this substance is directly proportional to the density of the cells in any local area and is indirectly proportional to the degree of stagnation in that area. In the body its formation is either prevented, or it is removed by the circulating blood. This extractable driving substance, or substances, of the cell, which acts according to its concentration, Burrows has named the "archusia." Its relations to cell migration, growth and reproduction have been summarized in another contribution (1926a).

In clean wounds of the adult skin, epithelium is the dominating tissue. Granulation tissue becomes excessive only when the epithelial cells are injured by infection or some other means. The dominance of the epithelial layer in the adult is not a function of age changes or any other peculiarity except the characteristic arrangement of epithelial cells in layers devoid of blood vessels, while, on the other hand, the lesser aggressiveness of the connective-tissue cells is related to their scattered distribution in a vascular tissue. In experiments with cultures it has been found that a dominating tissue may not only inhibit another tissue but actually destroy it and utilize it as food. Mesen-

chyme cells may thus grow at the expense of epithelial cells (Burrows, 1914) or the reverse (Barta, 1924). Furthermore, groups of cells prey upon other groups of their own kind as readily as upon cells of other kinds. In this regard is to be noted how the healing epithelium destroys in part, at least, the granulation tissue and so effects a secondary contraction of the wound.

Burrows (1924) accordingly concludes:

> Cell crowding and stagnation are the fundamental factors which regulate the normal balance between tissues, local hyperplasias and hypertrophies, atrophies, hyalinization, development, would healing and cancer. These differences in the end-result are due merely to quantitative differences in these fundamental factors. In the beginning of the life of the animal it has no circulation. It is with the gradual separation of cells and development of blood vessels that growth becomes irregular in the embryo, slows and ceases in adult life.

The growth characteristics of various adult tissues are due to their densely cellular nature, relatively impoverished circulation, or to both conditions. In wounds the growth is not for the immediate purpose of healing. It is usually excessive and beyond the bounds of the wound, corresponding to the areas suffering a reduction of circulation. Such reduction is the direct result of the primary changes which involve the destruction of blood vessels and the inflammatory reaction. The amount of growth is always proportional to the congestion and stagnation, but in the healing wound, as opposed, for example, to cancer, the primary changes are insufficient to prevent the return of a balance between cells and blood vessels.

VII. A THEORY OF WOUND HEALING. In order to understand the way of wound healing it is necessary to explain why it is that the various component processes proceed as they do. To consider adequately all the phenomena involved would transcend any reasonable limitations of space. Various aspects already discussed in previous sections cannot be repeated here. Instead a consideration of certain of the more obvious features will be reviewed, especially in regard to an interpretation already advanced by Loeb (1920a).

The building of a provisional granulation tissue, when it occurs, requires little explanation since it is essentially a filling process of embryonal material followed by gradual conversion into permanent connective tissue. Some of the activities of these cells are interpretable on the basis of theories to be discussed in later paragraphs. Retraction and contraction of the wound probably represent, for the most part, secondary (and in a sense accidental) features—however important they

may become from the practical, surgical point of view. Retraction is purely a mechanical phenomenon while contraction is surely due in considerable measure to the production and subsequent shortening of connective-tissue fibrils. This latter is a more or less secondary concomitant of connective-tissue reparation. Contraction, however, may transcend somewhat such a subordinate rôle if Burrows' (1924) contention be true that under certain conditions the epithelium actively drags the edges of the wound together. In this instance it would become a function of epithelial activity whose main phenomena are amoebism and mitotic proliferation. Turning, therefore, to these two most dramatic expressions of healing it is necessary to inquire further into the conduct of a differentiated tissue, epithelium, which by a process of lateral spreading is able to repair its defects. The problems of cell movement and proliferation are the same in essence whether they occur during wound healing, in the organization of blood clots, or in the growth of tissues *in vitro* (Loeb, 1919).

One important factor of amoeboid movement is the utilization of a stereotropic response by which cells (epithelial and others; Loeb, 1898) move always in contact with solid or semisolid bodies (Loeb, 1902; Burrows, 1913; Harrison, 1914). In the case under consideration the substrate is chiefly fibrin fibers. This stereotropic response explains the movement of epidermis along the floor of the wound, between scab and floor, or even along the fibers of the coagulum itself.[6] But there is apparently another agency at work which directs the cells away from their own tissue into the neighboring foreign material and especially into the wound defect.

> This activity finds expression in a centrifugal, often fan- or tree-like growth, which takes place in solid media in the absence of distinct directive fibers. This induces the various kinds of cells to move into clots and especially fibroblasts to organize blood clots; this induces likewise epidermis to fill the defect, and muscle and nerve to regenerate in a centrifugal direction (Loeb, 1919).

Much useful information on the problems at hand may be gained through observations on naturally free amoeboid cells. Loeb (1920) has supplied certain basic data through his studies on the amoebocytes of the arthropod, Limulus. When these cells leave the blood channels and come in contact with solid material their granular cytoplasm separates into a hyalin ectoplasm and granular endoplasm. The hyalin

[6] For a modification of the conventional concept of stereotropism, see Burrows, Burns and Suzuki (1917) and Burrows (1926b).

portion spreads outward in the form of pseudopodia, while at the same time the cell surface becomes sticky so that agglutination takes place between neighboring cells and between cells and the adjacent foreign body. Contact with solid substances, or other surface changes produced through chemical action, brings about alterations in the cell periphery which in turn leads to changes (physical and chemical) in the cell as a whole. It seems probable that these alterations of surface tension are preceded by metabolic changes, perhaps consisting of processes of ionization which extend progressively inward. Such cell ionization leads to water intake, increased viscosity, liquefaction and surface stickiness. Within physiological limits these changes may well be reversible. The ultimate factors underlying this process, the extent to which surface tension alone operates, and the degree to which the latter are mere accompaniments of more fundamental physical and chemical changes—all such desirable details must for the present remain unknown.

Unlike free amoebocytes, the cells involved in wound healing, clot organization and growth in tissue culture are rather definitely polarized to the effect of the foreign environment. The latter affects chiefly the side of the cell presented to it. As is well known, such polarized stimulation is accompanied by differences in electric potential on the stimulated side. This increase in potential may be deeply involved in the alterations in surface tension, and the accompanying physical and chemical changes, which characterize this side of the cell. Such potential differences will continue as long as a cell faces the foreign body in one direction and remains in contact with other cells of its kind in the opposite direction. Thus the conditions are set up which serve to direct a centrifugal movement toward the foreign body. Oxygen is a further requirement for this activity, and successful organization *in vivo* is dependent on the proximity of blood vessels. The rôle of an oxygen gradient as a directive influence is at present uncertain. In any event, by the production of lateral pseudopodia directed toward the foreign body the fan- or tree-like growth is brought about.

At the same time the changes in stickiness at the cell surface, already mentioned, lead to the characteristic stereotropic response. This reaction modifies the tendency toward strict centrifugal growth, and it is the stronger force so long as growth occurs where it may successfully operate. In this way deviation from a pure centrifugal growth is brought about. Nevertheless, in wound healing, stereotropism on the whole merely strengthens centrifugal growth. Thus it is that growth

continues until the effect of the foreign body is removed; that is to say, until the wound is closed and the clot organized.

There are certain other phenomena which are induced through the influence of a foreign substance. Cells which under these conditions are capable of movement can also manifest a phagocytic ability to a greater or less degree. This is not astonishing since phagocytosis is merely a further application of amoebism in response to the stimulating influence of foreign particles. That in both phenomena the same kind of factors are at work has been shown by several investigators (Levaditi and Mutermilch, 1910; Barakine, 1910). Another activity, often seen in wound healing, is the formation of giant cells; here the underlying principle is again the same. Marked alterations of the surface conditions of cells (probably as the result of induced physical and chemical changes, and combined with alterations of the electric potential) lead to an agglutination of the cells immediately surrounding the foreign body.

Of the responses induced in the regenerating epithelium there is left the important topic of augmented cell division. Loeb holds that apparently the same category of stimulating agents which induce amoebism and its allied phenomena also call forth a proliferative response in all those cells whose structure is not incompatible with such activities. How this comes to pass cannot be explained with satisfaction at the present time.[7] The internal structure and activities of the cell appear relatively more important in this reaction than the external influence (Burrows, 1927), yet the presence of a foreign body seems to be able to induce cell division.

Epithelioid cells may still divide mitotically as do the normal regenerating elements, but cells whose surfaces have undergone more profound changes and have transformed into giant cells exhibit amitosis. It is also known that cells living elsewhere in the body under unfavorable or specialized conditions respond similarly to the common stimulus that might otherwise be expected to induce mitosis. In the normal epidermis it is those cells which are farther removed from the body fluids that are suspected by some of indulging in amitosis.[8]

[7] The more detailed and analytic conclusions of Carrel and of Burrows as to the nature of the proliferative stimulus have been reviewed under "The Formative Stimulus" (p. 389 *et seq.*). Among the more recent theories which seek to explain the mechanics of cell division are the contributions of Lillie (1916) and Burrows (1927).

[8] The controversy as to the occurrence of amitosis in the epidermis has been renewed recently by Grynfeltt (1931), who is favorable to this interpretation, and by Mannes (1934) who is strongly opposed.

Thus, in the opinion of Loeb, the several reactions seen in wound healing may be considered essentially as the reactions of cells whose behavior is conditioned by the presence of foreign bodies—in the broadest sense of that term.

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CHRONAXIE

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The subject of chronaxie was reviewed in 1928 in this journal by H. Fredericq. He presented Lapicque's fundamental concepts of a time scale for the process of excitation and gave examples of the application of this scale to a wide variety of tissues. He also set forth Lapicque's theory of isochronism, which is based directly upon the concept of chronaxie. In the years following the publication of H. Fredericq's paper, determinations of chronaxie have multiplied in both laboratory and clinic, and a further theoretical superstructure, "*chronaxie de subordination*," has been added to the theory of isochronism. According to this recent development, the time factor in excitation becomes of major importance in the whole problem of the function and coördination of the central nervous system. At the same time, interest has been renewed in the fundamental theory of electrical excitation, and, with the recognition of the many variables, both inherent and adventitious, which are involved, the adequacy of the specific concept of chronaxie has been attacked. The wide divergence of opinion and the apparent contradictions in experimental observations demand a reconsideration of the entire subject. The present reviewers cannot settle questions of fact and may err in their evaluation of conflicting evidence, but they will endeavor to summarize these recent developments.

No attempt will be made to refer to all of the papers dealing with chronaxie. The comprehensive bibliographies given by Lapicque (1926, 1935b), H. Fredericq (1928), Rushton (1935), Monnier (1933, 1934), and others make this unnecessary, and until fundamentals are established it will be permissible to neglect details. Neither shall we attempt to review systematically the historical development of the subject previous to 1926, as it is well covered in Lapicque's monograph (1926). We shall consider the problem of electrical excitation. It will be obvious that none of the mathematical formulations can be considered entirely satisfactory. Therefore, as Lapicque has insisted for the last ten years, the index of the time factor of excitation must be essentially empirical, and we shall consider the merits of chronaxie as such an empirical index.

This is closely associated with the theory of isochronism, which we shall attempt to evaluate, and, finally, we shall outline the new facts and theory of "*chronaxie de subordination.*" Subordination, like isochronism, must stand upon its own merits as an empirical entity as long as its theoretical foundations are in question. Therefore, particular attention will be given to the experimental evidence relating to these concepts.

PHYSIOLOGICAL TIME. No one who has read Lapicque's monograph (1926) or his recent review (1935b) can fail to be impressed by his concept of "physiological time." This is the generalization that various processes, such as electrical excitability, muscular contraction, transmission of excitation, development of bio-electric potentials, etc., are essentially unitary in various tissues and follow similar laws when their activities are measured on appropriate time scales. Nerve impulses may travel at 100 meters a second or at 10 centimeters a second, but their mechanism is similar in all cases. "Fast" and "slow" tissues may differ ten-thousand-fold in their time factors of excitation and seem qualitatively different in consequence, yet, with adjustment of time scales, the fundamental similarity of the various cases is immediately evident, and the laws of electrical excitability become a generalization of the first importance. It has long been known from the early work of Fick, Hoorweg, Nernst, Weiss, Lapicque and Lucas that the duration as well as the intensity of an electric current is a determining factor in its efficacy as a stimulus. The briefer the current, the stronger it must be. If the threshold strengths of currents of varying durations are determined, a strength-duration curve may be drawn. Figure 1 shows the characteristic form of such a curve and also how the "chronaxie," the best-known index of the time factor in excitation, is derived from it. Chronaxie will be defined and discussed in detail below, but it is a relatively minor point whether this index survives as the most accurate and convenient guide to the various time scales. More important is the concept of time scales to be measured. Figure 2, taken from Lapicque's monograph, represents strength-duration curves of various tissues plotted in double logarithmic coördinates. This illustrates at a glance the fundamental similarity of the laws of excitation of very diverse tissues and the wide range of their time scales. All strength-duration curves are alike in that the necessary strength does not diminish indefinitely as duration is increased but reaches a lower limiting value (1R in fig. 1). This Lapicque has termed the "rheobase." *The "chronaxie" is the least duration of a current which will excite at a strength of double the rheobase* (C in fig. 1).

Likewise, whether or not we retain Lapicque's special theory of "isochronism," we can scarcely question the approximate similarity in (1) the time scales of tissues closely associated in function with one another, and (2) the speeds of various processes, such as excitation, conduction, and contraction, in a given tissue. As a first approximation, it seems

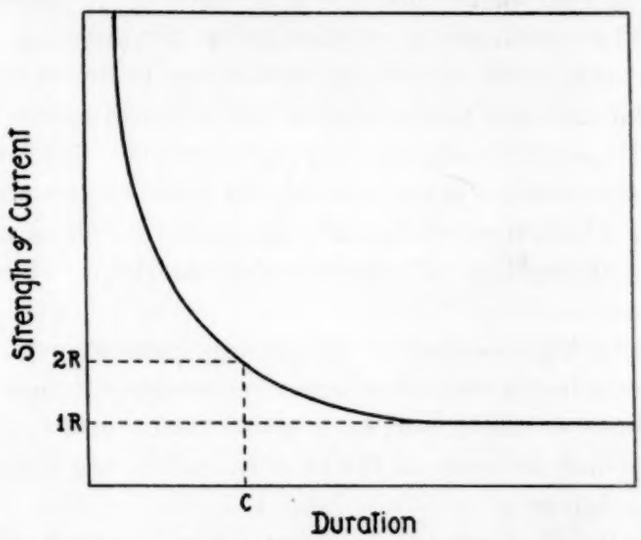

Fig. 1. Diagrammatic strength-duration curve of electrical excitability. 1R = rheobase. C = chronaxie (Lapicque) or excitation time (Lucas, Rushton). (After Rushton, 1935.)

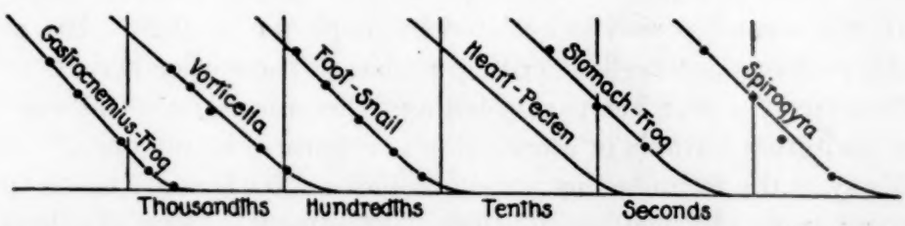

Fig. 2. Strength-duration curves of various tissues plotted in double logarithmic coördinates on a single extended time-scale, to show the similarity of behavior of fast and slow tissues. Most of each curve is a straight line whose slope is approximately ½. (The scale of ordinates is exaggerated in the figure.) (After Lapicque, 1926, p. 152.)

that a physiological system tends to be "fast" or "slow" as a whole, rather than fast in part and slow elsewhere.

Lapicque (1926) makes this generalization more precise, and proposes the chronaxie as the universal unit of physiological time. In these terms the law of velocity of nerve conduction becomes: "the impulse travels 10 cm. in one chronaxie"—a statement approximately true for all

nerves. Monnier and Dubuisson (1931) propose the term "*vitesse reduite*" for the speed expressed in this way, and offer a generalization as to how this "reduced speed" varies with the position of an organism in the phylogenetic scale.

EXCITATION FORMULAE. The validity of "chronaxie" as a measure of physiological time rests upon the generality of the laws of electrical excitation. The multitude of mathematical formulations which have been proposed attests at once to the divergence in detail of various sets of experimental data and to the hope of many investigators that all may be covered ultimately by a single law and formula. And it is upon the possibility of extending a given formula to other tissues and conditions than those for which it was originally devised, as well as upon its simplicity and its theoretical plausibility, that the value of each formula must be judged.

The present reviewers collected the various formulae which have been proposed to describe the empirical curve represented in figure 1. There were nearly thirty of these, and their presentation and individual analysis seemed beyond the scope of the present article, but a few generalizations may be of interest.

Chronologically the formulae fall into two main groups, the first beginning with that of Du Bois-Reymond in 1848 and including those of Hoorweg (1892), Weiss (1901), Lapicque (1903 and 1907b), Nernst (1908), and Hill (1910). Then follows a period of relative quiescence until the second period is initiated by Lapicque in 1926. His monograph contains an excellent critical review of the earlier period. Since 1926 scarcely a year has passed without the appearance of at least one new excitation formula or a revival in new guise of an old one.

Many of the formulae are purely empirical. Others are theoretically derived from physical assumptions. Prominent among the latter is Nernst's attempt, avowedly incomplete but dealing quite adequately with stimuli of short durations, on the assumption that the fundamental process of excitation is an electrical polarization. He likewise introduced the concept of an antagonistic phenomenon ("accommodation") to explain the divergence of his formula from the data for currents of relatively long duration. Lapicque (1926), with his "canonical formula," and others have attempted to deal with this antagonistic phenomenon, empirically or theoretically.

Another dominant idea has been the charging of a leaky condenser (cf. Hill, 1932). This leads to a formula for short durations which differs slightly from Nernst's. Both are of the type $i = kt^{-\alpha}$ where i is

intensity of current, t its duration, and k and α are constants. Alpha is expressed graphically by the slope of the lines in double logarithmic coördinates in figure 2. In Nernst's formula $\alpha = 0.5$. In the condenser type of formula $\alpha = 1.0$. Colle (1933) and Cole (1933 and 1934) independently pointed out that most sets of data fall intermediately between these two extremes and are fitted by values of α between 0.6 and 0.8. None lie outside the extreme values of 0.5 (Lapicque, 1934c) and 1.0 (Hill, 1935a, b). This suggests that tissues behave as intermediate between polarization capacities and fixed capacities.

Another conclusion that seems to emerge from the confusion is that if a formula is to embrace all of the reliable data on the great variety of tissues that have been investigated, then at least three parameters or fitting constants must be employed (cf. Colle and Delville, 1932). Hill's most recent formula (1935b) contains two independent time constants. Bonnardel (1934) employs an elaborate equation formally similar to that describing a bimolecular reaction. Apparently no simple theory or equation can cope with the complexity of electrical excitation, and any single index of the speed of the excitatory process must either be incomplete and limited in its application or be an empirical approximation. Lapicque's "chronaxie," with which we are primarily concerned, falls in the latter class (Lapicque, 1926).

Isochronism. *Lapicque's law of isochronism.* This law is the most specific and, if valid, the most important of the laws based upon the concept of physiological time. It rests upon the first generalization that the strength-duration curves of various tissues can be superimposed upon one another when the scales are suitably adjusted. Lapicque (1926, 1931a, b) believes this to be the case if suitable technical precautions are observed in the experiments, although Rushton (1935), Colle (1933), Simeone and Rosenblueth (1934) and others believe that the correspondence may be only approximate. Logically, the similarity in form of the curves is a necessary condition for comparison of the chronaxies (or other chronological indices) derived from them.

The law of isochronism states that within a single system, such as the somatic nerve-muscle preparation, the chronaxies of the successive portions of the conducting pathway do not differ from one another by more than a two-to-one ratio, and furthermore that this "isochronism" is a necessary condition for the transmission of excitation except in the case of "iterative systems." This theory has been invoked to explain, and is supported by, the action of curare and other drugs in blocking conduction from nerve to striated muscle. Lapicque (1906, 1926, 1934d) states that the

fundamental action of curare is to lengthen the chronaxie of the muscle while leaving that of the nerve unaltered, and that conduction fails when the ratio of chronaxies surpasses two to one. The thesis that isochronism is a necessary condition for conduction forms the cornerstone of Lapicque's (1907a, 1929, 1935a, b, c) theory of coördination within the central nervous system and lends significance to the *chronaxie de subordination*, which we shall consider below in greater detail.

The evidence for isochronism falls into two main categories. The first includes direct measurements of the chronaxie of striated muscle and its motor nerve, and the second is based upon the action of drugs, notably curare. Lapicque from the first has maintained that the chronaxies of the gastrocnemius muscle of the frog and its motor fibers in the sciatic nerve are the same. Keith Lucas (1907) found for the sartorius muscle of the toad an excitation time approximately ten times as great as that of its nerve. The followers of Lucas attributed the short values found by Lapicque to the stimulation of intramuscular nerve fibers, and Lapicque for many years did not seriously contest this particular point, basing his case upon his analysis of the action of curare. More recently he has repeated his early experiments in such a way as to obviate stimulation of intramuscular nerve fibers and has confirmed the equality of nervous and muscular chronaxies.

Size of electrode and the "α effect." Lucas placed the sartorius muscle in a bath of Ringer's solution and caused the current to act at one region or another on the muscle by placing an insulating barrier in the bath to concentrate the current flow locally in the muscle. When the pelvic end of the muscle, which is free from nerve-endings, was stimulated in this way, a single strength-duration curve, characterized by a slow excitation time, was found. When the mid-region, which includes nerve fibers, was stimulated, the curve was usually complex. It was composed of at least two branches, one of them corresponding to the slow excitability of the muscle (α) and another showing a fast excitability identical with that of the nerve (γ). In some preparations, Lucas also detected a third branch (β) which showed a still more rapid excitability. The β excitability was ascribed to the neuromuscular end-plates.

Lapicque regularly employed stigmatic electrodes, such as silver wires, and never obtained the slow excitability. Jinnaka and Azuma (1922) stimulated the pelvic end of the sartorius with small capillary electrodes and obtained a fast excitability. Davis (1923) showed that the size of the stimulating cathode was the significant variable in this situation, a small electrode giving the fast excitability and a diffuse electrode the

slow one. This relationship has been confirmed and greatly amplified by Watts (1924), Verryp (1924), Lapicque (1931b, 1932a) and others. A similar, but much smaller, effect has been found with nerve (Lapicque, 1931b).

The question naturally arose as to what, if anything, was the "true" chronaxie of skeletal muscle. If it be derived from the slow α curve, the law of isochronism is obviously invalid. This is also the case if we utilize the still slower "δ curve," which appears in the data of numerous workers from Lucas on, but was first clearly identified by Lapicque (1931b). Delta is observed in muscles immersed in Ringer's solution. It may be separated from α by a definite plateau. The rheobase is attained only with durations of 100 ms. (milliseconds) or more. This slow δ curve is abolished by the addition of a little extra calcium to the Ringer's solution. This leaves the α branch quite unaffected. Lapicque rejects δ as an obvious artefact.

Rushton has clearly identified the α excitability with muscle (1930, 1931, 1932a, b, c, d). All skeletal-muscle fibers show it, whether the muscle be intact or excised, if diffuse fluid electrodes be employed. With a diffuse cathode γ is associated with nerve, and the two excitabilities may be differentiated in this way (Rushton, 1935), although the presence of a local lesion or other "singular point" on the muscle fiber may give an unusually brief excitability (Benoit, 1934).

Lapicque emphatically refuses to admit that α is a truly physiological phenomenon. "The α curve has nothing to do with the chronological properties of muscle and is in no way a chronaxie." "The muscle excitability can be chronologically characterized only with small electrodes" (1933). Elsewhere (1926) he has insisted that only chronaxies derived from strength-duration curves which are "canonical"[1] can be accepted as valid. Since "chronaxie" is Lapicque's term we should accept these limitations upon it, and, for the general case use Keith Lucas' (1907) original term "excitation time," whose definition is identical with that of chronaxie, save for Lapicque's restrictions (Rushton, 1935). It is the duration at which threshold is reached with an intensity twice that required for very long durations (the "rheobase," cf. fig. 1).

[1] "Canonical" curves are those which conform to Lapicque's (1926) formula $i = a\frac{\sqrt{t+\theta+\sqrt{(t-\theta)^2+0.16\,\theta^2}}}{2t}$. Here i is the intensity of a liminal current, t its duration, a the rheobase, and θ a "constant of time" or "characteristic duration" equal to chronaxie $\times$ 3.8. The constant 0.16 is an empirical fitting constant. (See also Lapicque, 1931a.)

There are five arguments to justify Lapicque's rejection of α.

1. The α excitation time varies with the size of the electrode from a lower limit equal to the γ excitability up to a maximum apparently imposed by the available length of muscle fiber. The lower limit, equal to that obtained with stigmatic metallic electrodes, is reached with an electrode half a millimeter in diameter, although, in the case of capillary electrodes, it is also a function of the thickness of the wall of the glass tubing employed (Lapicque, 1932a). Rushton (1935), who obviously prefers fluid electrodes, admits that the choice of electrodes is arbitrary, and states that "whether one form of electrode should be used rather than another seems to be a matter simply of reliability and practical convenience. . . . If, as the electrodes are made progressively either larger or smaller the excitation time approaches some fixed upper or lower limit, such a limit might conveniently be taken as a practical value." Lapicque justly emphasizes the existence in fact of such a limit.

2. Two slow muscles with parallel fibers (the retractor of the head of the turtle and the longitudinal muscle of *Holothuria*) give α curves which are not at all different from the α curves of the frog's gastrocnemius, although, in respect to contraction and to excitability as measured by stigmatic electrodes in air, these muscles are 10 and 40 times slower, respectively, than frog muscle. In the case of *Holothuria*, the α and the γ curves are scarcely to be distinguished by their excitation times (Lapicque, 1933).

3. The α effect has a very small temperature coefficient. Benoit (1934) finds that the Q_{10} of the excitation time is well defined for a particular electrode of definite size on a given preparation. It is less than, or equal to, unity for the large constants obtained by fluid electrodes, while, for the γ excitability, a Q_{10} approaching 2 is found. The latter coefficient is consistent with that found for the chronaxie of nerve (Dworkin and Florkin, 1930) and by H. A. Blair (1935) for the constant, k, in his excitation formula. Blair cites previous data of Lucas and Mines, M. and L. Lapicque, and Filon as consistent with his own.

4. The α curves do not fit Lapicque's canonical curve. They conform instead to an exponential formula. The significance of this argument depends upon the evaluation of Lapicque's canonical curve, which is essentially empirical. In fact, Blair (1935), finding that Benoit's α curves fit his own exponential formula accurately, believes the shape of the curves is an argument in favor of and not contrary to their being "true physiological curves."

5. Curare has no effect upon the α curve. All investigators are agreed upon this point. Lapicque maintains, on the other hand, that curare systematically lengthens the "true" (γ) excitability of muscle, and Rushton (1935) agrees that it *may* do so.

Rushton and Lapicque both offer theoretical explanations of the α effect. Rushton believes that the α curve is fundamental and that γ in muscle is due to lateral diffusion of the ions which are being concentrated by the stimulating current. This effect would be important in a large tissue like muscle and insignificant in a small structure like nerve fiber. It follows readily from considerations such as those employed by Hill, Lapicque, and others in deriving excitation formulae that any such escape of ions should elevate the rheobase and shorten the chronaxie. Rushton has presented only a qualitative development of this idea, and it remains to be seen whether it will account for the phenomena quantitatively.

Lapicque, on the other hand, presents (1932a) the concept of "retrograde polarization," as follows:

> One must admit a polarization of the muscle surface under the cathode, starting from the edge at the point nearest to the anode, and developing itself progressively as far as the most remote point of the cathode. So, the current crossing the muscle surface is diverted backward all along the diameter of the cathode. In order to reach the threshold, the circuit must remain closed a sufficient time to allow (1) this polarization to come to its end; (2) the proper excitation process to be achieved. This sum appears in experiments as pseudo-chronaxie, evidently longer than the true chronaxie, and increasing *pari passu* with the length of muscle subject to the phenomenon.

He points out that this concept depends upon the assumption that the surrounding medium is a better electrical conductor than the substance of the muscle. He further states that "every condition and every phenomenon related to this retrograde polarization for the muscle is symmetrical to the physical electrotonus in the nerve, where, as classically and rightly admitted, the excitable substance (axon) is surrounded by a less conducting substance." He apparently considers it fundamentally related to the shortening of the chronaxie of nerve which is observed when the nerve is stimulated by two electrodes less than 10 or 12 mm. apart.

Lapicque's and Benoit's experiments on the size of electrode seem to be factually convincing, but we agree with Rushton (1935), who is "unable to obtain an exact idea of Lapicque's concept of the α effect and must refer readers to Lapicque's own account. It is clear that the α effect is supposed to be a polarization

which takes longer to mature the larger the electrode. The divergence [from Rushton's view] lies in the supposed effect of the polarization, for, whereas the physical theory assumes that this polarization results in excitation, following Nernst and others, Lapicque claims that the polarization must be complete before the normal and characteristic excitation process can begin." Lapicque evidently appreciates this difficulty implicitly, for he writes (1933, footnote 1 p. 398): "I must confess that I was surprised by this result, having admitted that a certain physical phenomenon which I called retrograde polarization [Lapicque, 1932a] is introduced between the moment when the electric current is applied and the actual stimulation. I thought that the sum of these two processes would always give longer durations than the actual stimulation time, in other words that the constant of α would always be longer than that of γ. To tell the truth, I had added that we were not dealing with a pure and simple addition of the necessary times for each process [Lapicque, L. and M., 1932b], and, on the other hand, that their complexity did not allow us to foretell theoretically the resultant of their conflict [Lapicque, L., 1932a, p. 274]. But if the theory of the α stimulation has become more obscure with the experiments on slow muscles, its physiological interest has also faded."

Before leaving the subject of false chronaxies, we must point out the grounds upon which Lapicque rejects Grundfest's (1932) measurements of chronaxie of individual nerve fibers. Grundfest excited individual nerve fibers in the retrolingual membrane of the frog by means of very small (20 to 60μ) capillary electrodes. He found that the strength-duration curve, and with it the chronaxie, varied with the distance of the electrode from the nerve fiber to which it was applied. The amount of connective tissue seemed to be a determining factor in this case. Lapicque contends (1934d), with some justice, that the physiological anode is very close to the physiological cathode in this case and that the values of the chronaxie are therefore fallaciously brief. In support of this view, he cites the data of Sakomoto (1933), who also employed capillary electrodes. Grundfest's values range from about 0.03 to 0.06 ms., and are in fact much shorter than the usual values for other nerves of the frog. It is noteworthy, however, that his values for muscle are in fair agreement with Lapicque's "true" chronaxie for muscle fibers, as indeed they should be with a stigmatic cathode.

Bishop (1928) and also Monnier (1934, chap. 15), as well as Grundfest (1932), raise the question of deformation of the stimulating current by the polarization of non-excitable tissues. With condenser discharges, for example, this distortion may be very different from that occurring with rectangular waves. The important question is *whether one is ever able to measure the absolute time factor in the excitation process itself when the depolarizing current is not applied directly at the seat of this process but*

through tissues which may alter considerably the effective duration and intensity of a given stimulus. Lapicque assumes that this distortion is negligible if the proper precautions are taken, but the critical question of its magnitude is still a matter of conjecture.

"Protoplasmic chronaxie" versus the "fiber-size rule." A cardinal feature of Lapicque's theory is the postulate that chronaxie is uniform throughout the extent of any given neuron (Lapicque, 1935b); that it is a property of the protoplasm associated with its permeability (Lapicque, 1934a, 1935b), and is therefore constant all the way from the dendrite through the cell body to the end of the axon. Each neuron is said to have its own natural "protoplasmic" chronaxie. Monnier (1934) has subscribed to this view in his interpretation of the findings of Lapicque (1912b) and of Gasser and Graham (1933), on which he bases his schema for the explanation of irreversible conduction in the reflex arc. This postulate seems to be a bold assumption unsupported by experimental evidence. In making it Lapicque has apparently abandoned the position taken in his earlier work with Laugier (1910) and with Legendre (1913, 1922) in which they emphasized the changes in chronaxie resulting from the action of various agents which changed the dimensions of the axon, a position emphasized by Lapicque (1926) when he observed a correlation of chronaxie with fiber diameter in the axons of a mixed nerve (cf. Lapicque, Gasser, and Desoille, 1925; Lapicque and Desoille, 1927). This correlation is accepted by Moore and Brücke (1931). Since a motor nerve fiber divides into smaller and smaller branches as it approaches the neuro-myal junctions (Cooper, 1929; Eccles and Sherrington, 1930), this correlation is clearly inconsistent with the doctrine of a uniform protoplasmic chronaxie more recently adopted. The correlation found by Blair and Erlanger (1933) between fiber diameter and velocity of conduction is in harmony with the earlier view, if we are to suppose there is a correlation between chronaxie and velocity of response. Some facts may be better explained on the assumption of a uniform chronaxie throughout the neuron, yet it seems quite as reasonable to suppose that the time parameter in excitation, as well as the velocity of response, may be determined in part by the form and dimensions of the excitable structure. If so, one might expect to find a different chronaxie in the dendrites from that in the perikaryon, and a different chronaxie from either in the axon, and still another in the terminal branches of the axon. Carefully controlled determinations on regenerated nerve fibers which have a smaller diameter than the parent axons (cf. Barron, 1933) would be of great interest.

Bourguignon (1933) from clinical observations on man, confirmed by experiments on rabbits, concluded that in certain pathological conditions the muscle affects the intramuscular part of the neuron, making its chronaxie different from that of its more central portions. These observations do not support the doctrine of uniform chronaxie throughout the neuron.

Experimental evidence for isochronism. We return to the evidence for isochronism. Support for an approximate isochronism is found in the general parallelism between chronaxie and various physiological velocities such as the speed of conduction and the rate of recovery from the refractory phase. Recovery in skeletal muscle is nearly but not quite as rapid as in medullated nerve. Conduction is roughly one-tenth as fast, and the duration of the rising phase of the action potential is about ten times as long, as in nerve. These figures are approximate (cf. Forbes, 1934), since no one has undertaken to measure these ratios directly in a single preparation. Nevertheless the ratio of one to ten probably indicates the degree of general isochronism which may be expected in a single physiological system.

A difference of one to ten in the time relations of the action potentials of nerve and muscle is much greater than a strict application of Lapicque's theory of isochronism would predict, since the chronaxies of skeletal muscle and its nerve are equal, and action potential is supposed to fit chronaxie, as key fits lock, within a ratio of two (or three) to one. It is evidently unsafe to make any such exact inference concerning another function from a knowledge of chronaxie alone.

Wachholder and Ledebuhr (1931) find that fibers of a given muscle differ from one another as much as ten-fold in their chronaxies although the excitabilities of the nerve fibers are quite uniform. The α effect seems to be ruled out, as they employed stigmatic silver-wire electrodes. They conclude that Lapicque's theory of strict isochronism is untenable. Now that the α effect is clearly recognized and can be avoided we may hope for further critical studies of this sort.

Another example of a function which reveals *approximate* isochronism between nerve and muscle may be found in the pararesonance curves described by Coppée (1934b) on these organs, using alternating currents. The relation between threshold intensity and the frequency is represented graphically by a curve presenting a more or less obvious minimum which shows the frequency of "pararesonance." This is a time characteristic which may be determined without any measurement of rheobase, but Coppée states that it is not sufficiently precise to be of practical utility. Nevertheless, the minimum point of the muscle curve is very close to that of its motor nerve.

Similar isochronism may be observed by considering a point on the steep portion of the curve, for example, the frequency at which an alternating current causes a muscular contraction at a potential (peak or effective) equal to the rheobasic voltage determined under the same conditions of circuit. This "isopotential frequency," as Coppée (1934a) calls it, bears a close and constant ratio to the chronaxie and is of the same order of magnitude whether determined on the motor nerve or on the corresponding muscle.

It is of some interest that the symmetry of Coppée's pararesonance curves plotted on semi-logarithmic coördinates disappears and the minima are displaced if he uses too short a distance between electrodes on the nerve or too large electrodes on muscle. We evidently face the issue of "true" and "false" frequencies of pararesonance.

Delville (1934a) utilized still another form of stimulating current, rising exponentially and either terminating abruptly when full intensity is attained or (1934b) immediately falling again exponentially. This method separates fast and slow excitabilities very effectively (cf. also Schriever, 1931, 1932, 1933). Delville's curves resemble those of Coppée in that the time characteristic appears as a minimum, independent of rheobase. Delville believes that he has demonstrated *heterochronism* between nerve and muscle, but it is quite possible that his "muscle" curves really are another manifestation of the "α effect."

Lapicque's theory of curarization. Lapicque's theory of isochronism is inseparably associated with his theory of curarization. He contends that curare lengthens the (true) chronaxie of muscle, and that conduction fails *when* and *because* it exceeds a two-to-one ratio. The recognition of the α phenomenon, which is not affected by curare, removes the basis of many recent criticisms of this theory.

Rushton (1933), however, performed one series of experiments in a manner which makes them comparable to those of Lapicque, and states that at the moment of failure of indirect excitability the chronaxie of the muscle remained unaltered. This, as Lapicque has emphatically stated, is in flat contradiction with his own twenty-five years of experience with the drug in hundreds of experiments. He suggests that the failure of indirect excitability in Rushton's experiments was spontaneous and not due to the curare. Such spontaneous failure may occur in spring frogs (cf. Dulière and Horton, 1929). This suggestion is possible, but Rushton does not accept it, since he never found any evidence of spontaneously impaired conduction either in these or in other comparable experiments. Further experiments would be of value in resolving the contradiction.

There remain, in addition, the experiments of Grundfest (1932), who determined the chronaxie of muscle in a bath of Ringer's, stimulating by means of a capillary cathode. The excitation times of his muscles appear to fall in the range of Lapicque's "true" chronaxies, although his curves

are not strictly canonical. He finds no change of muscular chronaxie as a result of the addition of curare, although indirect excitability fails. Lapicque has offered no satisfactory explanation of these experiments. On the other hand, Rushton's suggestion that Lapicque's increase of muscular chronaxie under curare is due to the calcium contained in this drug does not appear convincing (cf. Lapicque, 1934d). It certainly cannot explain similar findings with sparteine, which contains no calcium.

Lapicque has insisted upon the antagonism between curare, which lengthens, and veratrin, which shortens the chronaxie of muscle, as an important counterproof of his theory of curarization. Rushton failed in his attempts to confirm the existence of this antagonism (cf. Rushton, 1933), but he apparently accepts Lapicque's suggestion (Lapicque, 1934d) as to the reasons for his failure. The action of veratrin upon chronaxie is evidently far from simple. On the other hand, it would seem rash to found a theory of the generality of Lapicque's law of isochronism upon the evidence of this antagonism alone. Rosenblueth, Lindsley, and Morison (1936) point out that Lapicque's theory is inadequate to explain their recent observations on curarization and on fatigue. Rushton seems to be justified in his recent (1935) statement concerning Lapicque's theory of curarization: "It is clear that curarizations may be accompanied by a progressive lengthening of muscular chronaxie . . . it equally appears that this is not necessarily the case. It is to be hoped that work in the near future will reveal the conditions determining these two alternative results (as Lapicque has done for the strychnine-veratrine antagonism). In the meantime it would seem difficult to exclude the observations that in certain conditions curarizations may occur without chronaxie change, and hence the conclusion that conduction block cannot necessarily be due to this change."

In connection with certain apparent exceptions to his law of isochronism, Lapicque (1934d) makes a significant comment. He now thinks it "necessary, in a second approximation, to take into account rheobase side by side with chronaxie in curarization." If we are to accept the theory of electrical excitation of muscle by nerve or of one neuron by another, as presented by Monnier (1934), this consideration is inevitable. Monnier's treatment of the problem of curarization is particularly instructive. He shows theoretically that the problem of transmission between two cells is not symmetrical. Transmission should occur most readily, not when chronaxies are equal, but when that of the second element is *shorter* than that of the first. Therefore, although

block by curare is easily explained by the prolongation of the muscular chronaxie, the action of veratrin, shortening the muscular chronaxie, should be to *facilitate* transmission. The block actually observed must be explained otherwise, by its effect on the rheobase of the muscle. His interpretation of the action of strychnin is likewise at variance with the original view of Lapicque, but is supported by the experiments of Auger and Fessard (1933) and of Peugnet and Coppée (in press). Lapicque, in his insistence upon the importance of chronaxie and isochronism, had previously neglected the possible physiological significance of changes in rheobase. Monnier makes it clear that in this situation we must consider the strength and time-relations of the action potential of the motor neuron, which is assumed to be the stimulus to the muscle fiber. The chronaxie of the nerve is at best an indirect and approximate index of the time relations of the action potential. In the phenomenon of subordination we shall find (p. 427) another case of a change in excitability depending upon alterations in rheobase rather than in the time parameter. These considerations are fundamental in a theory of electrical transmission, but they rob the theory of isochronism of the primary importance originally ascribed to it by Lapicque.

We believe that the principle of approximate isochronism has a real descriptive value, but the experimental evidence and theoretical analysis do not justify the conclusion that strict isochronism is a necessary condition for neuromuscular transmission or that heterochronism is a sufficient or a necessary condition for neuromuscular block.

"ITERATIVE SYSTEMS," NEUROHUMORAL TRANSMISSION, AND ISOCHRONISM. The broad assumption on which the theory of isochronism is founded is that the action current of the neuron excites electrically the muscle or neuron upon which it impinges. This assumption is completely in accord with the "membrane theory" of the conduction of the nerve impulse (Bernstein; Lillie, 1922), which at present stands without serious rival. The membrane theory has been made the basis of interpretations of central nervous function by others than Lapicque (e.g., Lucas, 1917; Forbes, 1922), but however well established the membrane theory may be for conduction along the axon, its extension to intercellular conduction is still an assumption. The alternative theory of neurohumoral transmission has effectively replaced it with respect to the effectors of the autonomic system.

The autonomic effectors had always presented a serious difficulty for isochronism, for, in spite of uncertainties in the reported measurements (cf. H. Fredericq, 1928; Simeone and Rosenblueth, 1934; *et al.*), it was

universally agreed that the chronaxie of a smooth muscle was much longer than that of its nerve. The transmission of excitation in such "heterochronous" systems was explained by Lapicque on the basis of "*addition latente*" or summation of excitation (1910, 1912a, 1925).

Lapicque employed Richet's term "*addition latente*" to describe the case of smooth muscle stimulated directly by brief electric shocks. Although the tissue may respond to a single shock if it is sufficiently intense, the threshold is lower if a series of shocks are employed (L. Lapicque, 1912a). This, according to Lapicque, is typical of the general case of excitation by stimuli which are very brief in relation to the chronaxie of the tissue stimulated. Lapicque formulated certain laws relating the intensity and duration of stimuli to the number and frequency of stimuli necessary to elicit a response in such cases. Stimulation of *nerves* to various organs of long chronaxie, notably the effectors of the autonomic system, followed similar laws, except that, in general, a single shock, no matter how intense, was believed to cause no response of the end-organ. This latter proposition became the definition[2] of the "*nerfs iteratifs*" (Lapicque, 1926; Fredericq, 1928; Mazoué, 1930; Monnier, 1934). Such iterative systems were at once recognized as heterochronous, but no conflict with the theory of isochronism was involved as "*addition latente*" at the end organ could be assumed. As more refined methods of observation were employed, however, one after another supposedly "iterative" system was found to be "non-iterative" (e.g., *bladder*, Stewart, 1900; *stomach*, McSwiney and Robson, 1929; *nictitating membrane, iris*, Bishop and Heinbecker, 1932; *pilomotors, salivary gland*, Rosenblueth, 1932; *nictitating membrane*, Simeone and Rosenblueth, 1934; *crustacean claw*, du Buy, 1935). One might ques-

[2] This definition is unfortunate, since it involves the ability of an observer to detect a small response, and consequently the classification of many tissues has changed with improvement of technique. The other proposition, that the threshold is lower for repetitive than for single stimuli, would be still worse as a definition, since it would involve *a*, the ability of the observer to detect a small (threshold) response; *b*, the ability of the tissue to summate responses, which is usually a function of the mechanism of response, not of excitability, and finally *c*, an element of chance, i.e., the existence of differences in threshold among the fibers of the nerve under the electrodes. If all the fibers should have the same threshold (or the nerve should consist of only one fiber) no fall in threshold could be observed, because of the all-or-none character of nerve impulses. Change in threshold of the nerve is merely an indirect indication of differences in the *number* of fibers which must be excited to produce a visible response. Skeletal muscle can be made to appear iterative if a sufficiently crude indicator of threshold, such as movement of the limb, be chosen.

tion the present usefulness of the concept of "iterative systems" were it not that some effectors such as the uterus (Rosenblueth, Leese, and Lambert, 1933) show thus far not even an electric response to a single volley of nerve impulses. Certain spinal reflexes (Mazoué, 1930; Schriever, 1934) also meet the criterion, and Schriever makes use of the concept and the empirical laws of summation in an analysis of reflex function. On the other hand, inferences as to the chronaxie of central neurons (Mazoué, 1930) based on analogy with autonomic effectors are purely hypothetical.

The experimental evidence clearly shows that a non-iterative system may be heterochronous. This is probably true in general for the non-iterative autonomic effectors. Lapicque's theory of isochronism does not apply in these cases. But this limitation is perfectly natural, since Lapicque's isochronism is theoretically based upon electrical transmission, and it is now generally agreed that the autonomic effectors are activated by "neurohumoral transmission" or "chemical mediation." Lapicque (1934a) has tentatively identified the chemical mediators with his theoretical "state of excitation" already postulated in his theory of "*addition latente.*" There are physiological speeds, such as rate of decay, associated with chemical mediators, which determine the laws of temporal summation, but these are not chronaxies and should be measured directly in each case. Skeletal muscle is definitely non-iterative except possibly in the later stages of curarization (Bremer, 1932). The central nervous system is iterative in some cases, but usually not. Chemical mediation has not been established for these systems, but seems to be gaining support steadily, and the theoretical significance of chronaxie, obviously attached to the electrical theory, wanes in proportion.

"Subordination." Lapicque has suggested (1907a, 1911, 1929, 1935a, c) that his time measure of excitation—chronaxie—offers a clue to reflex coördination. An early application of this idea to irreversible conduction through the synapse was proposed by Lillie (1914) (cf. also Forbes, 1922, p. 373). The principle involved has been developed in more detail in a working hypothesis offered by Monnier (1934). He has shown that in nerve the ascending phase of the action potential is briefer than the utilization time and that therefore, if the action potential of one neuron be the stimulus for the next, synaptic conduction is best served, not by isochronism, but by a shorter utilization time in the second neuron than in the first. This arrangement, with suitable thresholds, would account for irreversible conduction. Monnier accepts the

inference of L. and M. Lapicque (1912b) that an element with large chronaxie exists in the reflex arc, and reinforces this inference with the observation by Gasser and Graham (1933) of a slow wave in the electric response of the spinal cord. Assuming chronaxie to be constant throughout the extent of each neuron, Monnier provides the requisite asymmetry for irreversible conduction by introducing two internuncial neurons with graded chronaxies leading to the motor neuron. His schema demands summation of afferent impulses to excite the first of the two internuncial neurons, which accords with the evidence of Sherrington (1929) and of Lutz (1918) (cf. Forbes, 1934, p. 183) that summation, either temporal or by convergence of paths, is essential to conduction through the reflex arc. Monnier's schema has the merit of plausibility in the light of his careful studies of the time relations in electrical excitation, but it cannot yet be considered more than tentative. In the form proposed it involves the assumptions of uniform chronaxie throughout each neuron and of electrical excitation at the synapse (cf. p. 417).

Lapicque, in 1907a, recognizing the need for something other than fixed pathways in the central nervous system, to explain behavior, suggested that a variable heterochronism between neurons would account for central coördination, and especially for the variability of patterns of response at the behest of the higher centers. The application of this idea required not only differences in the chronaxies of contiguous neurons, to determine which should be excited by the afferent impulses, but some power to change the relative values of these chronaxies in order to emancipate behavior from the limitation of a fixed pattern of response.

In 1923 M. Lapicque observed a change of chronaxie in the frog's sciatic nerve when this was severed from the spinal cord. This appeared to reveal an influence of the higher centers on the time factor in the excitation of motor neurons. Bourguignon (1923), stimulating the motor points through the skin in man and in dogs, had already found in synergic muscles the same chronaxie but large differences between the chronaxies of antagonists. Usually the extensor motor nerves have a chronaxie about twice as large as the corresponding flexors. This difference is said to be abolished either by cutting the motor nerve, by transecting the spinal cord, or by anesthetizing the animal (Lapicque, 1935a). Rudeanu and Bonvallet (1932a, b, d; Bonvallet and Rudeanu, 1932) found that both in the case of anesthesia and of operative procedures the equalizing of diverse chronaxies was correlated with disappearance of reflex coördination. Modifications of chronaxie have also been reported as resulting from proprioceptive stimulation, induced by

passive flexion or extension of the hind limb under observation (L. and M. Lapicque, 1924; Rudeanu and Bonvallet, 1932c). Rudeanu and Bonvallet state that the two-to-one ratio of extensor to flexor may actually be reversed by such manoeuvres. Jasper (1932a, b) also reports that the difference in chronaxie between extensors and flexors is greater on the right side in right-handed persons and on the left side in left-handed persons. He has found a similar difference in rats, which could be reversed by application of cocain to the dominant cerebral cortex (left cortex in right-dominant rats, and *vice versa*) (Jasper and Bonvallet, 1934).

All these experiments are taken by Lapicque and his school to show the presence of an influence emanating from the nerve centers, which changes the chronaxie of the motor neurons. When not subject to this influence, a neuron is said to have a "*chronaxie de constitution*"; when the influence acts, this is changed to the "*chronaxie de subordination.*" The effect of subordination was originally described as a reduction of chronaxie, as shown by the fact that, when it is abolished by any of the procedures above mentioned, chronaxie becomes longer. More recent experiments (Monnier and Jasper, 1932c) have shown that the most characteristic effect is to cause a highly variable chronaxie, either above or below the stable level which is reached after suppression of the activity of the centers. According to Lapicque (1935a, c) the effect emanates chiefly from a center in the mesencephalon (probably the red nucleus) in mammals, and in frogs chiefly from centers between the cerebrum and the medulla, for a section below this level is said to abolish it. Yet according to M. Lapicque and Vahl (1931) and M. Lapicque (1932c) subordination may be found in the spinal frog with small doses of caffein or strychnin, and Jasper and Bonvallet (1933) have found evidence of subordination in the spinal rat, correlated with the recovery of reflexes after transection. Lapicque argues that subordination is not essential to machine-like regularity of response, but confers the variability of response which characterizes the influence of the higher centers. He contends that, in absence of the center for subordination, the reflex patterns in the spinal cord are largely fixed.

Perhaps the most revolutionary aspect of subordination as described by these workers is the implication of a disturbance other than a nerve impulse conducted over peripheral nerve trunks from the central structures where it originated. The difficulty thus raised and the possibilities of reconciling the phenomenon with the known properties of nerve function will be discussed later. For the present it will suffice to note

the caution with which the evidence should be scrutinized before final acceptance.

Lambert, Skinner, and Forbes (1933) investigated the possibility of explaining the changes ascribed to subordination as a consequence of the local effect of cutting the nerve. They found that when they cut the motor nerve of a decerebrate cat or frog within less than about 2 cm. of the stimulated point a sharp increase in chronaxie resulted. This was correlated with a decrease in rheobase, and it was noted that the changes in both chronaxie and rheobase corresponded with those described by M. Lapicque (1923) as a consequence of severing the motor nerve (cf. Cardot, 1912; also Monnier, 1934, p. 271). They found little or no effect on either chronaxie or rheobase when the motor nerve was severed 3 cm. or more away from the point of stimulation. They therefore ascribed the effect to the injury in the vicinity of the stimulating electrodes. Complete strength-duration curves taken when nerve section had induced large changes in rheobase and chronaxie revealed little or no change in the time relations; the curve was merely shifted vertically, as was shown by the almost perfect superposition of the curves before and after section when a fixed voltage increment equal to the drop in rheobase was added to each point. When a change in the time parameter occurred, it was usually in the opposite sense to the change in chronaxie. These authors were thus led to the hypothesis that the lowered rheobase or shift of baseline of the curve was due to a partial stimulation of the nerve by the injury current. Taking duration measurements at fixed increments of voltage above the rheobase in order that the curves might be more directly compared as to the time factor, they repeated a number of the experiments which had been reported by other workers as revealing the phenomenon of subordination. Neither section of the spinal cord in decerebrate animals nor anesthesia nor proprioceptive stimulation produced any significant changes in time parameter, nor any consistent changes in rheobase or chronaxie. They therefore concluded that some of the evidence for subordination had depended on the proximity of section to the point of stimulation and the consequent lowering of rheobase due to the injury current.

This explanation is obviously inapplicable to the changes following sections in the central nervous system remote from the site of stimulation. In appraising the latter type of experiment they noted that Lapicque and his co-workers (L. and M. Lapicque, 1924, 1928; Jasper, 1934) found subordination only in active animals in very good condi-

tion, and described it as unsteady and subject to rapid fluctuations. Since the active state of an animal is apt to result in shift of electrode contact on the nerve, and since a shift of contact is apt to change the apparent threshold and to deform the lines of current flow through the active tissue, Lambert, Skinner, and Forbes (1933) suggested that changes in chronaxie under these conditions might not signify a real physiological change.

Tawast-Rancken (1933) reported observations on rheobase and chronaxie of the motor nerve in frogs under anesthesia and after section of the spinal cord and subsequently of the motor nerve itself. Anesthesia caused the same effect found by M. Lapicque (1923), viz., decrease in rheobase and increase in chronaxie. Spinal-cord section caused small changes in the same sense, which were only transient. Nerve section 13 mm. from the electrodes caused much larger changes, similar to those found by Lambert, Skinner, and Forbes, being in the same sense as the Lapicque effect, but subject to partial recovery. Apparently effects emanating from the higher centers, although small compared with those related to local injury, do exist.

At the same time that these investigations were in progress, Monnier and Jasper (1932a, b, c, d; Monnier, 1933) were engaged in experiments which have done much to throw light on this baffling problem. Their experiments were carefully controlled to guard statistically against deception by chance fluctuations. They avoided the effect of injury current by sectioning the motor nerve a long distance from the stimulating electrodes. These experiments appear indeed to bring out a real effect which cannot be ascribed to injury current due to the proximity of the nerve section to the point of stimulation.

The most important feature of these experiments is the revelation that the "subordination" effect is essentially a change in rheobase and not in the time parameter of excitation, that the change in chronaxie is purely secondary to the change in rheobase in the same manner that has been found in the case of sections close to the stimulus [ascribed by Lambert, Skinner, and Forbes (1933) to injury currents], and that in almost all of these respects the effect of subordination closely resembled that of anelectrotonus and is probably ascribable to increase in polarization of the cell membrane (cf. L. and M. Lapicque, 1924). Monnier (1933) has compared the changes in nerve found under subordination with those found by Bishop and Erlanger (1926) and other workers under anelectrotonus and has tabulated the results as follows:

	SUBORDINATION	ANELECTROTONUS
Rheobase	+	+
Chronaxie	−	−
Rheobase × chronaxie	Constant	Constant
Velocity of conduction	−	−
Amplitude of action potential	No appreciable change	Slight increase
Duration	No appreciable change	Slight increase
Demarcation potential	+	+
Absolute refractory period	−	−

The interpretation is given graphically in the curves reproduced in figure 3. The correspondence is indeed striking and strongly suggests the existence of an increase in polarization under those conditions in which subordination has been reported.

Monnier (1933) also mentions certain instances in which "*subordination*" has appeared in the opposite sense, suggesting an effect similar to catelectrotonus. He suggests that these opposite effects may be correlated with the opposite central effects recognized as inhibition and facilitation in the spinal reflexes.

In recognizing that subordination is an effect on rheobase and not on the time parameter of excitation, Monnier (1934, p. 258) justifies the use of chronaxie for a measure of the effect as a means of distinguishing between a change in rheobase involving the excitable mechanism and a change due to the introduction of an external resistance into the circuit. A change in observed rheobase from such an external cause is not accompanied by any change in chronaxie, whereas a change in true rheobase, such as is found in subordination, is regularly accompanied by an inverse change in chronaxie; hence the need of recording both quantities. Thus it should be emphasized that in concluding that subordination is a polarization effect which alters the *rheobase* of the nerve, but leaves the time factor in excitation unchanged, *the rôle originally assumed for chronaxie in the coördination of the nervous system is abandoned.*

A perplexing aspect of this phenomenon of subordination is the question of its origin. Fredericq (1932) reported changes in chronaxie of the left visceral nerve of the cephalopod *Eledone moschata* which he ascribed to effects emanating from the innervated heart muscle, without participation of higher centers. He calls this effect "metachronose." More recently (1933) he has reported a similar phenomenon in the

vago-auricular system of the turtle and the sciatic-gastrocnemius preparation in the toad.

The ultimate nature of subordination is a baffling question. In spite of its elusive nature and of the negative findings of some observers, there seems to be enough positive evidence to warrant the conclusion that a real effect, of the sort analyzed by Monnier, exists. It is difficult to reconcile such an effect with the known functional properties of nerve. When a nerve trunk is stimulated—no matter how—a nerve impulse of the all-or-none type, qualitatively unvarying, is propagated away from the point of stimulation. The observed phenomena of subordination

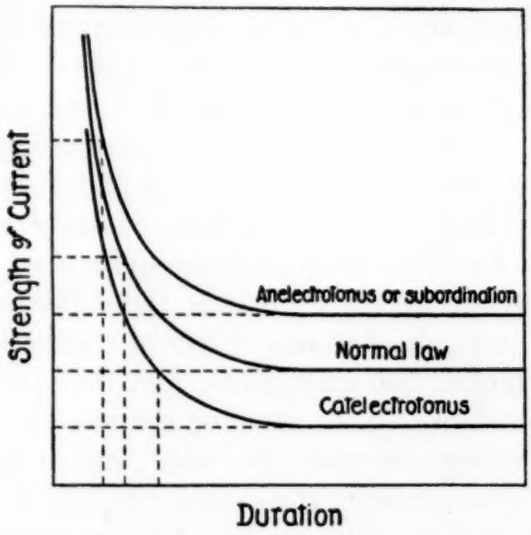

Fig. 3. Diagram to illustrate changes in strength-duration curves of electrical excitation induced by electrotonus, involving rheobase but not the time parameter. Dotted lines indicate the determination of chronaxie in each case. (After Monnier, 1933.)

imply the ability of the nerve fiber to conduct another sort of disturbance quite distinct from the nerve impulse. An explanation in terms of the known properties of the nerve impulse would simplify the picture. This would be attained if we could suppose that under conditions of reflex activity which evince subordination nearly all the motor fibers accessible to threshold stimuli are being kept busy conducting a rapid succession of impulses and are therefore in either the absolute or the relative refractory phase. This simple explanation is probably ruled out by the relatively low frequency of motor neuron discharge in all but the most vigorous reflex contractions (Adrian and Bronk, 1929).

Achelis (1928) and Brücke (1935; cf. also Brücke and Krannich,

1931) arrive at the conclusion that reflex subordination is a real phenomenon, possibly mediated in some way by the sympathetic nervous system.

If, as the evidence of Monnier and Jasper seems to show, the effect is an increase in polarization resembling that caused by anelectrotonus, it must either arise from electrical potentials set up in the nerve centers or from some chemical substance acting on the cell membrane. Barron and Matthews (1935) have recently described an effect appearing in the white matter of the spinal cord, for which they consider the most probable explanation to be an electrotonic effect resulting from the potential gradient arising in the adjacent gray matter. But in the case of the axon of a peripheral nerve trunk, a similar explanation seems highly improbable, for even complete severance of a nerve, involving presumably the greatest possible depolarization, does not appear to influence the characteristics of excitability for more than about 2 cm. from the section. It seems, therefore, impossible that any physiological potential gradient in the nerve centers could cause a measurable polarization in the nerve trunk 4 or 5 cm. away from the spinal cord (much more than this in mammalian preparations), where the subordination effect has been detected. Such an electrotonic effect should be much greater near the center than at the extremities, and this difference should be capable of measurement. If we abandon the idea of electrotonic effect arising from potential gradients in the center, and turn to the spread of material from the centers along the axons, it is very difficult to see how this could take place with sufficient speed to produce the rapidly fluctuating effects which have been described.

The recent work of Feldberg and Gaddum (1934) and the evidence adduced by Rosenblueth (1934) have made it appear likely that conduction across the synapse in the reflex arc is effected by some chemical mediator. If this is indeed the case, we can imagine effects produced in the center by liberation of even subliminal quantities of the mediator at the synapse, but how such effects could cause a measurable change to spread rapidly to the peripheral part of the neuron is as difficult to conceive as in the case of electrotonus arising in the center. In absence of any tangible evidence, we must for the present remain in the dark as to the essential mechanism of this effect. The mass of experimental evidence attesting the existence of subordination, coming from several laboratories, must be taken seriously. Yet the elusive and fluctuating character of the phenomenon, rendering quantitative measurement difficult, and the obscurity of its essential nature, combine to make the

cautious critic desire still more rigid proof before accepting it as conclusively established.

If the fundamental nature of subordination is obscure, so also is its significance as an element in reflex function. We have shown that the theory of chronaxic switching as depending on changes in the time parameter of excitation is not tenable in the light of recent evidence. Whether the changes in polarization, which seem to be established as the true change involved in subordination, play a significant part in modifying reflex behavior is also a matter of conjecture.

CHRONAXIE AS AN INDEX OF SPEED. Since Lapicque's theory of isochronism has failed, the significance of chronaxie rests largely upon its validity and convenience as an index of the "speed" or "time scale" of various tissues (cf. p. 409). The difficulty of assuring ourselves experimentally of "true" chronaxies is an argument against it. It is then pertinent to inquire whether another index may be preferable. It is by no means certain that a single general index can be found, since the relation between speed of excitation and speed of conduction, for example, need not necessarily be the same in all tissues.

It has been tacitly assumed that all fibers innervating a single organ, such as gastrocnemius muscle or nictitating membrane, would have nearly the same chronaxie, but Rosenblueth and Rioch (1933) find a systematic variation, first a fall, then a rise, as the chronaxie of nerve fibers of higher and higher threshold innervating the nictitating membrane is measured. Wachholder and Ledebuhr (1931) find a similar variability in skeletal muscle. This introduces a new element of uncertainty into the situation. In the past, practically all determinations have been performed on the most excitable fiber, but recently Hill and his school have adopted methods which instead yield indices of the *mean* fiber (Scott, 1934; Bugnard and Hill, 1935). They believe that the indices so obtained are more precise, characteristic, and more reproducible than those obtained by threshold methods.

Blair and Erlanger (1933) surveyed the entire population of fibers in one of the small nerves of the frog's toe, and have examined the relations between their various measurements, which included fiber diameter, velocity of conduction, refractory period, duration of rising phase of the action potential, and chronaxie. All except chronaxie show a satisfactory correlation, increasing or decreasing together or inversely. Chronaxie deviates systematically in the case of the A fibers, and they consider it the least satisfactory index for the prediction of the value of the other functions. In fact, velocity of conduction

and absolutely refractory period may well prove more satisfactory general indices, and we believe that for the speed of the excitation process itself a single satisfactory index has not yet been found.

As an "index of excitability" Lassalle (1928) has proposed the reciprocal of the product (rheobase^2 x chronaxie). This has the dimensions of energy, and may be of some value in expressing relative changes of excitability in a given tissue. For comparisons between different tissues it suffers from all of the uncertainties involved in measuring both rheobase and chronaxie, but, since it represents energy rather than time, it seems more logical than chronaxie as a *single* index of "excitability."

We conclude that chronaxie may be useful as a rough measure of the time factor in excitation in comparing different tissues. The way in which it has been shown to vary under influences which do not change the true time parameter renders it unsuitable as an accurate measure of the latter. The measurement of chronaxie is fairly simple, and it has come greatly in vogue. Numerous clinical studies have suggested various interesting laws (cf. Bourguignon, 1933). Their utility and significance are purely empirical and they must be carefully and critically tested before being accepted (Schriever, 1930). Bean (1934) shows how difficult it may be, even in animal experiments, to meet the various conditions for reliable determinations. Bonnardel and Liberson (1934) occasionally encounter what seems to be an "α effect," even when using stigmatic electrodes according to Lapicque's specifications.

Many workers in both clinic and laboratory believed that in measuring chronaxie they were getting a true measure of "excitability," which replaced the determination of threshold by previous methods. Also, unfortunately, many measurements have been made without adequate discrimination. Changes in chronaxie have been described in a complex system as bearing on the end-organ when the measurements were made upon the innervating nerve. For instance, many workers have measured chronaxie, applying the stimulus to a nerve and observing the response of the innervated tissue, e.g., muscle or vascular reflex center. They have then applied drugs or other agents acting on the center or effector, and reported the apparent change in chronaxie, ignoring the fact that the stimulus was applied to the nerve, which was not affected by the agent they were studying. The fallacy of this plan of research is not hard to see. It is like measuring the hearing of a telephone subscriber by the number of coins which must be dropped in

the slot to establish a connection. If chronaxie is to be used as a practical measure, such limitations as this and others already discussed should be clearly understood at the outset. Chronaxie as a scientific concept should not be forced to bear the onus of bad experimentation.

SUMMARY

Tissues vary widely in their speeds of activity, but various processes in a given tissue, such as bioelectric phenomena, conduction, contraction, and excitation, may, as a rule, be appropriately measured on the same time scale. If the time scales are properly chosen, the laws describing these processes apply to a great variety of tissues.

The chronaxie, selected by Lapicque as the moxt convenient measure of the speed of excitation, has been widely employed as a "measure of excitability" without due regard for its arbitrary and empirical character. Originally identical with the "excitation time" of Lucas, it is now restricted to determinations made with certain types of electrodes and under conditions which yield a "canonical" strength-duration curve. Neither the canonical formula of Lapicque nor any of the many other theoretical or empirical formulae which have been proposed have proved entirely satisfactory. The phenomena of excitation appear too complex to be characterized adequately by a single simple chronological index.

An *approximate* isochronism usually exists between a skeletal muscle and its motor nerve. Lapicque's theory of strict isochronism and his associated theory of curarization are not substantiated by the experimental evidence or by theoretical analysis, in spite of the fact that Rushton's major criticisms have been met by the recognition of the "α effect." The latter depends on the sizes of excitable fiber and of electrode. Isochronism does not seem to be a *necessary* condition for neuromuscular transmission, nor heterochronism for "curarization."

The effectors of the autonomic nervous system, whether "iterative" or "non-iterative" are apparently excited by a neurohumoral mechanism. Since the theory of isochronism is based on the assumption of an electrical mechanism of transmission, it should not be applied to the autonomic effectors. Empirically these systems are heterochronous.

Explanation of central nervous function on the basis of isochronism and of "subordination" is purely speculative. The evidence for subordination, i.e., a change in the characteristics of peripheral nerves dependent on influences other than nerve impulses emanating from the central nervous system, is reviewed. The changes do not appear to

involve primarily the time factor of excitability, but seem to be akin to anelectrotonus. The theoretical significance of these changes is obscure. The demonstration of the phenomenon experimentally depends upon a recognition of its characteristic variability and of its sensitivity to the state of activity of the centers.

The use of chronaxie as a clinical index of excitability should be placed upon a completely empirical basis, and as much attention given to standardized determinations of the rheobase as to the chronaxie itself.

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THE RÔLE OF VITAMIN C IN THE ORGANISM AS SUGGESTED BY ITS CYTOLOGY

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Within the last two years international workers have given an entirely new outlook to our knowledge of the physiology of vitamins by their isolation, and synthesis of vitamin C and their investigation of its relation with the various organs of the body.

Szent-Gyorgyi's (49) (50) original discovery in 1928 of a strongly reducing substance in the adrenal cortex was really the forerunner of researches which did not finally eventuate in their full vigour for another four years. Szent-Gyorgyi extracted this reducing substance from the adrenal and regarded it as an acid of the Hexuronic series.

Tillmans (58) subsequently made a suggestion that the distribution of Szent-Gyorgyi's hexuronic acid in nature coincided with the distribution of vitamin C. Szent-Gyorgyi and Svirbely (45, 47, 48) then succeeded in curing scurvy in guinea pigs with this hexuronic acid, and King and Waugh in Science (37) and in the Journal of Biological Chemistry (38) stated that they had obtained from lemon juice crystals of a substance which showed antiscorbutic properties and was similar to hexuronic acid in its chemical and physical properties.

The next step was to ascertain that this hexuronic acid was vitamin C and that the real vitamin C was not an impurity in the acid.

Harris (23–32) of Cambridge, Svirbely and Szent-Gyorgyi (49–56), and Cox (10, 11, 12) and others worked on this subject and Haworth (35) established that its chemical nature did not make it a true hexuronic acid and the name was therefore changed to "ascorbic acid."

Most of the details of the research associated with the final establishment of ascorbic acid as vitamin C and the synthesis of the compound first by Haworth and secondly by Reichstein, Grüssner, and Oppenauer (42) are now well known and will not be repeated here.

Haworth has recently announced that he has elaborated a synthetic process which will make the cost of pure synthetic crystalline vitamin C only a few pence a gram, and the door appears open to a new era in physiology.

Probably one of the most important phases of this vitamin C work was the discovery that vitamin C occurs at a high concentration in the cells of the adrenal gland, the corpus luteum, the interstitial cells of the testis, the anterior pituitary, and, not so concentrated, in the liver.

Moore and Ray (40) were the first workers following Szent-Gyorgyi, to conduct macroscopic staining tests of the adrenals. They found the adrenals of normal guinea pigs blackened very rapidly with silver nitrate, whereas the adrenals of guinea pigs which had been for some time on a scorbutic diet were completely unaffected by the reagent. They found, unlike Szent-Gyorgyi with the ox adrenal, that the medulla reacted just as strongly as the cortex. The first microscopical descriptions of the vitamin in the gland cell were given by the present author in a paper to Nature in 1933 (2) while Giroud and Leblond and other workers from the Paris school published a series of papers on the cytology of vitamin C in various organs during 1934 (13–22).

In December 1933 the present author (4) showed that the adrenal gland of the human fetus contained vitamin C and a discussion was also given on the physical state of the vitamin in the gland cell.

A certain diversion of opinion occurs as to the exact form of the vitamin in the cell. The original preparations of the present author were made after a preliminary fixation of the tissue in formalin vapour, but owing to the fact that a proportion of the vitamin C was destroyed by the fixative, this method was soon abandoned and a method employing alcoholic silver nitrate substituted. Further preparations were made by freezing the fresh tissue and dropping the frozen sections into acidified silver nitrate. Giroud and Leblond's technique consists in washing out the blood vessels of a freshly killed animal with isotonic levulose solution, then injecting silver nitrate. The tissues were subsequently fixed with photographic "hypo," as were my own preparations, and sections cut.

Giroud and Leblond found the vitamin C granules to be in the form of small granules or short rods. They stress the similarity of the preparations to those of mitochondria. They found no tendency towards perinuclear condensation of the granules and in addition they stated that there was no association of the vitamin C granules with the lipoid droplets of the adrenal cortex. In addition they made the statement that only very rarely does the zona glomerulosa show the presence of these vitamin C granules.

I have found the most common form of vitamin C in the adrenal to be finely granular with varying degrees of perinuclear condensation.

Leblond also considered that Moore and Ray, and the present author, and Siehrs and Miller (44), are in error in describing blackening of the medulla with silver nitrate. Harris (32) has shown that the medulla may contain its vitamin C in a reversibly oxidised condition, in which state while still biologically potent, it will not reduce silver nitrate. Not all the adrenals examined by the author show a silver nitrate reducing medulla, quite a number of them failing to react. But it is of utmost importance that Breidahl and I (5) have found that the adrenal medulla following anaphylactic shock, chloroform and ether anesthesia, anger or other strong emotional reaction has its vitamin C in reducing condition, in which case it gives a very strong reaction with silver nitrate—much stronger than the cortex. The conditions mentioned above all cause a strong defense reaction in the medulla—i.e., a liberation of adrenalin. The liberation of adrenalin from the adrenal medulla, therefore, is accompanied by an alteration in the vitamin C oxidation-reduction system whereby the reversibly oxidised vitamin is again brought to the reducing condition.

What is the significance of this change? Is it necessary that the vitamin C should become reduced before adrenalin can be liberated, or is the reduction an effect of the liberation of the hormone? One finds an aggregation of fine granules of vitamin C around the nucleus in the medullary cells, which do not occur in animals killed rapidly by a blow on the neck, etc. These granules appear in the same site as that in which the granules of adrenalin make their first appearance and would suggest that they either assist in the synthesis of the adrenalin granules by the cell or prevent their oxidation as they are formed. It appears then that vitamin C is bound up intimately with the working of the adrenal medulla, probably in the direction of the synthesis of adrenalin. Whether this is so or not, it is of considerable importance that the nervous impulse to the medulla is capable of activating the vitamin C oxidation-reduction system either directly, or indirectly through the medium of acetyl chlorine. If vitamin C really is bound up with the production of adrenalin then its relation with what appear to be adrenalin deficiency diseases, such as asthma, is very obvious.

Vitamin C in the cortical cells takes many forms. I have obtained preparations similar to those described by Giroud and Leblond, I have obtained preparations in which the granules of vitamin are very finely divided but the granules show little perinuclear condensation, or again similar preparations in which a great deal of such condensation occurs. All degrees of aggregation of the vitamin may occur, from the

fine state of division just mentioned, to relatively large masses of argentophile material which may or may not be in association with the nucleus.

The degree of dispersion of the vitamin is without doubt related to its activity in the cell and the degree of perinuclear condensation appears also to be some significance, for in the adrenals of pregnant rabbits it was found that the perinuclear condensation became more marked as pregnancy advanced. When the vitamin occurs in a finely dispersed condition it would indicate that chemical reactions of the cortical cell dependent upon the vitamin are proceeding rapidly; when the vitamin occurs in masses then it would indicate a slowing up of such activity (Alexander (1)—Zone of maximum colloidality).

Leblond in his preparations found no relation between the lipoid droplets of the adrenal cortical cells and the granules of vitamin C. A number of my preparations have, however, shown this. Perhaps the most outstanding preparation was one of the adrenal gland of the human fetus (50 cm.) (6). In this gland it was found—first, the granules of vitamin C occurred chiefly in the fetal cortex; second, they exhibited a considerable amount of perinuclear condensation—the nucleus being completely obscured in many cases; third, each granule was not entirely composed of vitamin C, but possessed a central core of lipoidal material.

Then in preparations of the adrenals of other animals varying stages in the association of vitamin C granules with lipoid droplets were observed (3). Some of the droplets showed the vitamin C in the form of black bands surrounding each side of the lipoid droplet, in others this band was continuous right round the droplet, in others two or three large blobs of the material were observable around the droplet. Then all stages were observed between this condition and one in which the vitamin was in the form of very fine granules completely surrounding the droplets. In some preparations no association of the granules with the lipoid droplets were observed and the general appearance of the cell was very similar to that described by Leblond, that is, rodlets and granules of vitamin C scattered among the lipoid droplets but not in association with them. The association of the granules of vitamin C with the lipoid droplets therefore appears to be dependent upon the functional state of the gland cell.

In a recent publication (6) I showed that the golgi apparatus appeared intimately associated also with the lipoidal droplets, but some pains were taken to ensure that the granules of vitamin were not confused

with golgi remnants. In the human fetus it appears that the lipoid droplet undergoes its genesis under the influence of vitamin C.

What the exact relation is between vitamin C, the golgi apparatus and the lipoid droplets of the adrenal cortical cell will require further investigation before it is solved.

The cortical hormone of Hartman (34), Swingle and Pfiffner (57) and Rogoff and Stewart (43) which has recently been isolated in a pure crystalline condition by Doctor Kendall (36) of the Mayo clinic who is so ably endeavouring to synthesise it, is apparently a fat soluble substance, and there appears little doubt that it is bound up within the lipoid droplets of the adrenal cortical cell. What, then, is the significance of the association of vitamin C with the lipoid droplets? May it play some part in the genesis of the cortical hormone? Its chemical nature, its ability to combine with glutathione (known to be present in quantity in the cells of the adrenal gland) in an oxidation reduction system makes it an ideal promoter of syntheses not only in the adrenal gland but in other organs as well.

Hartman and his co-workers (33) have found that if cortin, carefully prepared so as to contain no vitamin C, was given to guinea pigs on a scurvy producing diet, the onset of scurvy was delayed.

Hartman considered that cortin was therefore associated with the utilisation of vitamin C by the organism. It is rather difficult to appreciate how the cortin could utilise the vitamin C when the animal was on a scorbutic diet. It seems much more likely that cortin is *one* of the factors associated with the clinical manifestations of scurvy and that vitamin C is intimately bound up with its synthesis.

Gough and Zilva (23) first showed that vitamin C was contained in large amounts in the pituitary gland and shortly afterwards (5) Breidahl and I showed that in scurvy, while the adrenal lost entirely its ability to reduce silver nitrate after two weeks on a scorbutic diet, the pituitary stained as intensely as before. Svirbely showed that in scurvy the liver was one of the first organs to lose its vitamin C content thus indicating that it acts mainly as a storehouse of the vitamin.

Leblond (39) has shown that only the chromophil cells of the pituitary contain the granules of vitamin C, and the chromophobe cells do not contain any at all. I am able to support this statement from my work on the pituitary glands of rats, guinea pigs, rabbits, mice and various marsupials.

It is of interest that Zilva first observed a blackening of portions of the ovary with silver nitrate and subsequently Huszak (35a) found by

chemical and biological means that the corpus luteum contained as high a percentage of vitamin C as any other organ in the body.

In the guinea pig I have found that the germ cells show only a slight reaction with silver nitrate. In the normal ovary, not only in the guinea pig, but in the human being and in the cat and dog it was found very difficult to pick out definite interstitial cells. In every case where the ovary contained a corpus luteum, however, these cells became much more obvious. It is difficult in the guinea pig to produce a well defined corpus luteum with the luteinising principle of the anterior pituitary gland, but it is possible to cause a luteinisation of certain of the cells. Normally the cells of the ovary react very poorly with silver nitrate, but immediately they show signs of luteinisation they commence to react strongly with this substance. The granules of vitamin C in these cells tend to exhibit a perinuclear condensation and usually surround any lipoid droplets present. The interstitial cells which also become obvious when the lutein cells appear, also exhibit a strong reaction with silver nitrate.

Leblond (39) has found in the cells of well defined corpora lutea that the vitamin C occupies a similar form and that there is a similar tendency for these granules to be aggregated in the centre of the cell around the nucleus. He is impressed by the mitochondrial appearance of these granulations in the cell.

What is the function of vitamin C in the corpus luteum? Mouriquand and Schoen (41) have found that pregnant animals cannot be given scurvy. I have been unable to give scurvy to my guinea pigs with lutein cells in the ovaries. It is known that the very young infant is capable of synthesising its own vitamin C and it appears highly probable that the fetus does so also. But apparently there is a very great need for additional vitamin C during pregnancy for it appears that the corpus luteum also is capable of synthesising it.

The cells of the anterior pituitary, the corpus luteum, the adrenal cortex and the interstitial cells of the gonads usually contain large amounts of lipoidal droplets, they frequently contain lipochrome pigments and they almost invariably contain large amounts of vitamin C.

All these organs are intimately bound up with sex changes (virilism may result from disturbance of function of the adrenal cortex) and it is of very great importance that vitamin C occurs in all these sites. What is the significance which lies behind this fact? At the moment it is very difficult to say. But one thing is immediately apparent, that is, the great need for an adequate supply of vitamin C in the diet. Szent-

Gyorgyi at the recent British Association meeting has laid a number of diseases at the feet of a slight chronic vitamin C deficiency and as research progresses it appears that many more diseases will be found to be due to the same cause.

The only other organs I have examined have been the kidneys and I have found the vitamin C to be in the form of small granules and rodlets occupying a position between the nucleus of the cell and the lumen of the uriniferous tubule. Apparently they represent droplets of the vitamin going through the process of being excreted into the urine.

Leblond (39) has examined the other organs and tissues poor in vitamin C such as the intestine, but for further details one can refer to his excellent little monograph.

In conclusion it seems that vitamin C is of very profound importance to the organism. What exactly its function is, we cannot say at present. Leblond quotes evidence in favour of its function as part of the respiratory action of the cell. Whether this is so or not I feel that its ability to form oxidation reduction systems together with the very significant associations one finds in cytological preparations makes it highly probable that it is intimately bound up in cellular syntheses particularly in the adrenal and anterior pituitary and perhaps the corpus luteum and interstitial cells of the gonads as well.

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ELECTROLYTES IN MUSCLE

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Since muscles involve a large fraction of the body weight, the discussion of muscle electrolytes involves also all the larger questions of body electrolytes in general. This subject has been reviewed recently by Peters (1935) and the distribution of water in the body has been reviewed by Adolph (1933). An effort has been made to limit this review to problems more closely related to the muscle alone. Another aspect of the subject which has been omitted concerns the modifications of muscle function (excitability and contractility, etc.) caused by variations in concentrations of electrolytes. This review is concerned only with the rules which govern the movements of water and electrolytes in and out of the muscle cell.

OSMOTIC RELATIONS. An isotonic solution may be defined (for a muscle) as one in which the muscle neither gains nor loses water. Actually this definition does not usually apply strictly to any solution for the muscle first gains some water and then loses it more slowly, so that after a certain special interval of time it may appear to have retained its original weight. Moreover, even if its water content is unchanged its electrolyte content may be appreciably different. The definition, therefore, becomes of purely empirical significance. It suggests, however, that the muscle cell is freely permeable to water which enters if the osmolar concentration is too small outside and leaves if it is too large (Overton, 1902a; Cooke, 1898; Loeb, 1898). A gain of water by the whole muscle does not, of course, mean necessarily a gain of water by the cells, as will be made clear in discussion of the tissue spaces.

In spite of this permeability to water the cells in the body retain a more or less constant water content and remain in equilibrium with their immediate environment. This is not difficult to understand if all osmotically active substances can freely penetrate the cell, but it seems necessary to believe that the cell proteins, at least, are not diffusible and that the colloid osmotic pressure of a muscle cell, containing 20 per

cent protein must exceed that of the intercellular fluid (partially deproteinized plasma) just as the colloid osmotic pressure of the red blood corpuscle exceeds that of the blood plasma. To explain a water balance in the presence of a non-penetrating osmotic fraction it is necessary to assume: 1, some force involving a continuous expenditure of energy (as in secretion processes); or 2, some mechanical force (turgor) such as the hydrostatic pressure in the capillaries which balances the colloid osmotic pressure of the blood; or 3, a fixed total electrolyte content of the cell due to total impermeability to all ions or to impermeability to either cations or anions. Other possibilities must certainly be exhausted before secretory properties are ascribed to muscle membranes in general. A difference in hydrostatic pressure across the sarcolemma, if present at all, must be exceedingly small compared to the osmotic pressures concerned. The cell is not surrounded by a rigid wall like the cellulose wall of plant cells nor has it the appearance of a cell surrounded by an elastic membrane which is inflated like a balloon. If one end of the muscle fiber is cut off the remainder is not suddenly deflated but continues to function normally. If then the sarcolemma does not maintain a turgor pressure inside, the osmotic balance must be maintained in general by an impermeability to electrolytes, either to cations, to anions, or to both, although there may, of course, be functional changes in the membrane to complicate the hypothesis.

Duff (1932) has endeavored to measure the colloid osmotic pressure of muscle. Some objections might perhaps be raised to his method and his suggestion that this pressure is balanced in vivo by a cellular turgidity has already been questioned. In any event the low value of about 45 mm. of mercury which he obtained amounts to only about 1 per cent of the total osmotic pressure of the muscle interior, and, at 20 per cent protein inside the cell, indicates a reasonable molecular weight of 76,000.

When muscles are removed from the body and are exposed to abnormal solutions, their permeability may undergo changes. If they are normally cation-permeable and anion-impermeable they may become also anion-permeable in their new environment to a greater or lesser extent; electrolytes may thus be enabled to enter along with the water which goes in because of the unbalanced osmotic pressure of non-penetrating anions inside. Under such conditions a muscle will continue to swell in any solution however hypertonic it may be. This explains the fact that frog muscles will continue to take up water from a 20 to 25 per cent potassium chloride solution (v. Fürth, 1919). The mem-

brane apparently resists moderate hypertonicity without disintegration and the muscle shrinks.

Some such increase in anion permeability may also help to explain why mammalian muscles require a definitely hypertonic solution, 1.4 to 1.5 per cent sodium chloride (Freund, 1904; Vandervael, 1934) for the maintenance of weight. Even frog muscle is reported to gain weight for a time from solutions of sodium chloride usually called isotonic (Overton, 1902a; Schwarz, 1911; Vandervael, 1934; Bucciardi, 1932; Gentile, 1934). Over a 5-hour period some muscles gain and some lose water in Ringer's solution. The typical behavior seems to be an initial gain in weight followed by a loss (Schwarz, 1911), the loss being coincident with a decrease of irritability (Bucciardi, 1932).

It is probable that the movement of water between the inside and the outside of the muscle fiber is determined by the osmotic pressure difference but attempts that have been made to prove this have not been very successful. Loeb (1898) found that the change of weight of muscles immersed in hypotonic and hypertonic solutions was not proportional to the difference in the osmotic pressure of the solutions. Thus the ratio $\frac{\Delta \text{ weight}}{\Delta \text{ concentration}}$ tended to decrease with increasing concentration. This does not indicate, however, that the volume changes of the muscle are not explainable in terms of osmosis alone, as Loeb implied, for, according to the osmotic theory, the product of volume and concentration at equilibrium should be a constant, $VC = V'C' = K$. In this case $\frac{dV}{dC} = -\frac{K}{C^2}$, i.e., $\frac{dV}{dC}$ decreases in inverse proportion to the square of the concentration approximately as Loeb found.

Overton (1902a) tried to ascertain whether the equilibrium volume which a muscle attains after equilibration with a hypotonic or a hypertonic solution could be predicted from the osmotic pressure of the solution. After allowing 20 per cent for the volume of the tissue spaces which he assumed to be unchanged by the solution, he found that the volume change was less than would be expected and suggested that some of the water in the muscle was bound water. If there is no leakage of electrolytes into or out of the fibers during immersion the relations should be expressed by the equation $(V - b)C = (V' - b)C'$, where C and C' are the concentrations of two different solutions and V and V' are the corresponding volumes of the muscle fibers after allowing for the tissue spaces. In the author's laboratory, the value of b has been calculated in this way, the volumes of the tissue spaces being determined

from the chloride content of the muscle as described later. The value of b turned out to be about 35 per cent of the initial weight of the muscles. Since about 23 per cent of the initial weight of the fiber is solid dry material the result might be taken to indicate that some 12 per cent of the muscle is bound water, or water which does not participate in the osmotic exchange in the time allowed. The values of b so obtained, however, are not very constant, probably because the electrolytes do not remain constant within the cell.

A somewhat similar study of weight changes of muscles was made by Hill (1930). From the difference in weight between two muscles, one in hypotonic $(1 - r)$ and the other in hypertonic $(1 + r)$ Ringer, he calculated the fraction of the muscle which was osmotically active and found that it amounted to 47 per cent of the muscle. Since the total water is 77 per cent, this meant that 30 per cent of the muscle water was osmotically inactive. This might be the extracellular water plus water contained in dead or injured cells which had become completely permeable to electrolytes, and which should contain chloride in the same concentration as in the outside solution. The author has found, however, (unpublished) that the chloride content of such muscles is too small to account for all the osmotically inactive water. Rather than assume the existence of any bound water in the muscle it seems simpler to believe that the electrolyte contents of the two muscles do not remain equal during the period, thus invalidating the calculation. Some complicating factors are certainly involved, for the muscle in the hypotonic solution gains water for a time only and then begins to lose weight before the gain is complete.

Other indications of the predominant importance of osmotic pressure in the movement of water are found in the work of Kőrösy (1914) who showed that isotonic solutions of glucose, sucrose, sodium chloride, and potassium chloride had equal effects on muscle water. Variations with other solutions are met with which may be attributed to effects upon the muscle membrane and which cannot be properly evaluated. Better results are obtained, perhaps, by considering only initial rates of water movement and Adolph (1931) has found that this initial rate is a linear function of the osmotic pressure of the solution, except in the most dilute solutions where an abnormally rapid gain in weight may indicate some injury to the membranes. While the movement of water is therefore chiefly due to osmotic differences between the inside and the outside of the fibers, the situation is complicated by varying volumes of tissue spaces, diffusion of electrolytes and modifications or injuries to the muscle membranes.

Composition of frog muscle. Some further evidence for the equality of osmotic pressure inside and outside the muscle fibers is afforded by a study of the osmotically active substances known to be present in frog muscle and plasma as listed in table 1. The table shows particularly a comparison between the composition of frog muscle (sartorius and other small muscles) and plasma as found by analyses made in the author's laboratory. Some analyses by other writers are included for comparison. Similar figures are given by Ernst and Scheffer (1928). Hill and Kupalov (1930) have made a somewhat similar comparison on the basis of analyses by Katz (1896) and by Meigs and Ryan (1912) but omitted the nitrogenous constituents. By the measurement of vapor pressure they found that frog blood is isosmotic with a solution of 0.731 gram of sodium chloride per 100 grams of water and they assumed that this figure must represent also the osmotic pressure of the muscles. Table 1 shows that plasma has a total known concentration of anions plus cations of 240 millimols per kilo of water while the "isotonic" sodium chloride solution has a concentration of 250 millimols per kilo. Ringer's solution (with phosphate) has a similar concentration of 249 millimols per kilo. A comparison of muscle and plasma on the same basis shows that both contain 240 to 247 millimols per kilo of water. Comparison of osmotic pressures is not strictly permissible in this way without a knowledge of the activity coefficients of the various constituents and of the ionic strength of the various media, but at least the comparison shows equality within the limits of the uncertainty of the method. An attempt to calculate the ionic strength inside a muscle fiber was made by Fenn and Maurer (1935).

A noteworthy feature, evident in table 1, is the large excess of cation equivalents over anion equivalents in the muscle. This is generally true in plants as well as in animals (Jacques, 1935). The difference is partly made up of proteins. There are missing 47 m.-eq. of anions per kilo of muscle in a total of 130. According to Weber (1934) the muscle proteins in 1 kgm. of muscle between pH 6.4 and 7.3 can combine with only 26 m.-eq. of base. It is difficult to believe that this is much underestimated, for at 17 per cent protein this would give an equivalent weight of the protein anions of 654 and Weber (1927) estimates 815 for myogen as an anion. There is left, therefore, 21 anion equivalents unaccounted for. If carnosine and amino acids were added in as anions it would account for 18 more milliequivalents, but carnosine is described as a base (Clifford, 1921; Baumann and Ingvaldsen, 1918) while the amino acids over the physiological pH range are probably mostly in the

TABLE 1

Osmotically active constituents of frog muscle and plasma

(Millimols per kilo fresh tissue)

	MUSCLE				PLASMA	RINGER	0.731% NaCl	Cl SPACE (14.7%)	K SPACE
K	73.0[U]	89.5[M]	79.0[K]	83.0	2.5	1.3	0	0.4	82.6
Na	14.2[U]	23.5[M]	23.9[K]	25.4	103.8	123.0	125	15.3	10.1
Mg	8.2[U]	12.3[M]	9.5[K]	8.3	3.0	0		0.4	7.9
Ca	2.2[U]	7.0[M]	4.0[K]	2.5	2.0	1.8		0.3	2.2
Cl	12.7[U]	18.6[M]	11.3[K]	10.9	74.3	115.9	125	10.9	0
HCO_3			7.0[H]	10.7	25.4	0		3.7	7.0
Lactate			3.0[H]	2.8	3.3	0		0.5	2.3
Hexosemonophosphate, etc.			1.9[E]	6.1					
Phosphocreatine			21.4[E]	11.1					
Adenosinetriphosphate			4.8[E]	3.2					
Orthophosphate			4.6[E]	12.8					
Total phosphate			(32.7)	(33.2)	3.1	6.7		0.5	32.7
Carnosine			11.0[E]	11.0[E]					11.0
Creatine			6.1[E]	1.7	2.1			0.3	1.4
Urea			1.7[E]	1.6	2.0			0.3	1.3
Amino acids (?)			6.8	6.8	8.5			1.3	5.5
Water, ml. per kilo			816[K]	(800)	960	1,000	1,000	140	660
Anion equivalents			77	82.6	108	126	125	15.9	65
Cation equivalents			130	130	166	126	125	17.1	113
Millimols per kilo water			240	247	240	249	250	242	248

Analyses were made in the author's laboratory except those indicated by an initial letter. The authorities so referred to are as follows: U = Urano (1908a), M = Meigs and Ryan (1912), K = Katz (1896), H = Hill and Kupalov (1930), E = P. Eggleton (1935). The author's figures for creatine, urea, and amino acids (Fenn, 1935) were obtained from analyses of Ringer's solution with which the muscles had been equilibrated for 5 hours, these substances being freely diffusible. The amino acids were analyzed as amino nitrogen by Van Slyke's manometric method. It was presumably not carnosine, which is indiffusible. It is assumed that lactate, creatine, urea and amino acids have the same concentration in the plasma water as in the muscle water. In calculating the cation and anion equivalents the phosphates were considered to have an average valence of 1.75, it being impossible to make a more accurate estimate without knowing the pH. This figure was based on Eggleton's calculation for pH 7.0 as quoted by Hill and Kupalov (1930). The hexosephosphate fraction is calculated to include all the acid-soluble phosphorus not accounted for in the other groups. The last four substances were omitted from the calculation of the equivalent concentrations. Figures for Ringer and sodium chloride were calculated. The Ringer's solution consisted of 0.65 per cent NaCl, 0.02 per cent $CaCl_2$, 0.01 per cent KCl and M/150 phosphate buffers (85 per cent Na_2HPO_4 and 15 per cent NaH_2PO_4). Other similar figures for frog plasma are given by Urano (1908a), Fahr (1908), and Chistovich (1931).

form of zwitterions (Van Slyke and Kirk, 1933). As a further difficulty it may be mentioned that the interior of the muscle is probably appreciably more acid than pH 7.0 (cf. Fenn and Maurer, 1935) so that the protein anions are probably overestimated rather than underestimated.

This anion deficit may depend upon variations in the magnitudes of the phosphate fractions. In gastrocnemius muscles, particularly, the author has observed larger values than those given in table 1 (50–55 millimols per kilo) which would completely make up the anion deficit. These analyses differed from those in table 1 chiefly in the larger values for the fraction including hexose-monophosphate. Netter (1934) quotes figures which indicate a complete balance between anions and cations without including any protein anions, his acid-soluble phosphate value being 50 millimols per kilo instead of 33, as in table 1.

TISSUE SPACES. One great difficulty encountered in the study of muscle electrolytes is the presence of tissue spaces and the necessity for estimating the electrolytes present in the intercellular fluid, the lymphatics and the blood vessels. Overton (1902a, b) recognized this difficulty clearly and estimated that 20 per cent of the muscle volume was accounted for in this way. He further conceived of the perimysium as being very slightly elastic so that as the muscle swelled in the hypotonic solutions the increased elastic pressure squeezed out some of the extracellular water, the gain in weight being entirely intracellular. Conversely, he believed that muscles which had shrunk in hypertonic solutions were harder to dry with filter paper because of the increased amount of extracellular water permitted by the diminished extension of the perimysium. This was also said to have been verified microscopically. Based though it was upon inadequate evidence, this conclusion was partly confirmed in the writer's laboratory by the finding that the chloride space after soaking is, in general, less in slightly hypotonic and more in slightly hypertonic, than in isotonic solutions (unpublished). Some exceptions occur, particularly in hypotonic solutions, due possibly to penetration of chloride into injured fibers.

Hermann (1888), with the aid of Langendorff, measured, in frozen cross sections of frog sartorius muscles, the width of the tissue spaces as 1 where the diameter of the fibers was 12.3. Hence the tissue spaces may be estimated roughly as $\frac{(13.3^2 - 12.3^2)}{13.3^2} \times 100$ or 14.5 per cent of the total volume. In some similar sections kindly prepared for the writer by Dr. I. Gersh[1] a tissue space area of 17.5 per cent was found by the

[1] The muscles were frozen, dried in vacuo without thawing, and embedded in paraffine. The cutting of the sections was kindly supervised by Dr. G. W. Corner.

use of a planimeter on camera lucida tracings. This was the smallest value obtained, but it was probably too large rather than too small.

Actually, none of the histological observations seem perfectly convincing and it is possible to believe that in the normal muscle there are no tissue spaces of appreciable magnitude except for the blood vessels, lymphatics and the various membranes.

Fenn, Cobb and Marsh (1934) have shown that if all the chloride of frog muscle were confined in 14.7 per cent of the total muscle it would have a concentration equal to the chloride concentration of the frog blood plasma. The close agreement between this value and the minimum volume of tissue space observed histologically lends strong support to the theory that the "chloride space" is the same as the intercellular space. This indicates that none of the chloride of muscle is present inside the cells and that the volume of the tissue spaces can be estimated quite accurately by the ratio of muscle chloride to plasma or solution chloride. This hypothesis is further strengthened by the finding of Urano (1908a, b), Fahr (1908) and Ernst and Takács (1931) confirmed by Fenn, Cobb and Marsh (1934) that muscles in isotonic sugar rapidly lose all their chloride through the electrolyte-permeable perimysium. The latter authors showed further that the chloride content of muscle tended to be directly proportional to that of the solution in which the muscles were soaked. Hastings and Eichelberger (1935) have also concluded that none of the chloride of muscle is present inside the cells (see also Peters, 1935).

When estimated on the basis of the chloride contents of blood and muscle the tissue spaces, or chloride spaces, of mammalian adductor muscles, as measured by Winter (1934), were 14.5 per cent in the rat, 15.5 per cent in the dog and the guinea pig, 12.5 per cent in the cat, and 22.4 per cent in the hen, all very similar to frog muscle. Corper (1915) injected NaSCN into rabbits and subsequently determined its concentration in blood and muscle, the latter value being 13, 16, 9.2 and 9.2 per cent of the former in four determinations. These figures agree closely with the similarly calculated chloride space for intact muscles and probably refer to the same thing. This may be taken as further indication that the muscles are anion impermeable.

An independent estimate of the volume of the tissue spaces in frog muscles has been made by Schulze (1927) who found a very high value of 36 per cent. The method depended upon the measurement of the conductivity between electrodes placed upon the muscle a known distance apart before and after soaking the muscle in isotonic sugar solution.

The decrease in conductivity was assumed to be due entirely to the loss of a column of fluid of specific conductivity equal to that of Ringer's solution. Knowing the length of this column the cross sectional area could be calculated and was taken as the cross section area of the tissue spaces. The formula used by Schulze was apparently incorrect for a recalculation from the same data gives an average value of 13.9 per cent for the tissue spaces.

Urano (1908a, b) believed that the tissue spaces of muscle were measured by the sodium content of the muscle rather than the chloride content, but was unable to show always that all the sodium came out into isotonic sugar solution. Höber (1922, p. 472) and also Hastings (1933) have estimated tissue spaces as 20 per cent on the basis of their sodium content. If it is true that all the sodium of muscle is confined to the extracellular fluid with the chloride, then the high Na/Cl ratio in muscle becomes difficult to explain.

Meigs (1912) estimated the tissue spaces of frog stomach from histological examination as 15 to 20 per cent of the total and found no change after immersion in Ringer.

Fenn, Cobb and Marsh (1934) and Fenn (1935) have shown that the chloride space of isolated muscles goes up from 14.5 to about 33 per cent after immersion in Ringer's solution. This probably represents in part an increase in tissue space at the expense of the muscle fibers. It may be due in part also to the death of some fibers on the outside of the muscle which then lose their potassium and take in sodium chloride. The loss of potassium from muscles in Ringer may possibly be taken as evidence of damage to some of the fibers (Horton, 1930). Similarly in soaked muscles, M. G. Eggleton (1933) found that 20 to 30 per cent of the muscle could come into equilibrium with phosphate in the solution and 16 to 27 per cent could come into equilibrium with glucose (M. G. Eggleton, 1935). Conway and Kane (1934a, b) found the chloride space 36 per cent, the phosphate space 35 per cent, and the sulfate space 28 per cent, diffusion of these anions into this extracellular space being practically complete in 15 minutes. The much more complete diffusion of the lactate ion which they recorded may be interpreted perhaps to mean that it could penetrate as undissociated lactic acid rather than as lactate ion.

From these observations on the isolated muscle there emerges the theory that the chloride content of tissues is a measure of the volume of the tissue spaces, i.e., the space which is in free diffusion equilibrium with the blood for both anions and cations (except colloids). From this

point of view the total chloride content of the body becomes a quantity of considerable interest. An early value given for this is that of Magnus-Levy (1910) who found 123 mgm. chloride per 100 grams body weight in man. At a plasma concentration of 104 m.-eq. per liter this leads to the calculation that 39 per cent of the body is chloride space. In a study of the excretion of creatinine, Dominguez, Goldblatt and Pomerene (1935) have calculated an equivalent plasma volume in the body amounting to 23 to 30 per cent of the body weight, which is probably closely analogous to the chloride space of the body. Crandall and Anderson (1934) injected 5 per cent NaSCN intravenously and measured its subsequent concentration in the blood. The result showed that it had diffused freely into 32.5 per cent of the body, this being another measure of the chloride space. A similar interpretation is given by Peters (1935). The rôle of chloride in the body has recently been reviewed by Irving and Manery (1936).

Acting upon the assumption that extracellular water of muscle is measured by the chloride content, the electrolyte content of the chloride space of frog muscle has been calculated in table 1 by taking 14.7 per cent of all the electrolyte concentrations found in plasma and assigning 140 cc. of the 800 cc. of muscle water to it so that the concentration per kilo of water will be the same in the plasma and in the tissue spaces. Strictly the lack of protein in the tissue spaces should be allowed for but this would not materially change the argument. Deducting the tissue space electrolytes from the total contained in the muscle the contents of the potassium space or the cellular space is thus obtained as given in the last column. This leaves almost all the potassium inside the fibers and all the chloride outside. It may be observed that the excess of cations over anions is very large inside the fibers. It is evident furthermore that the total molecular concentration is such inside the fibers that if any considerable fraction of the potassium is bound in any indiffusible form the osmotic pressure inside would be too small to balance the osmotic pressure of the plasma.

Since the bicarbonate content of plasma is over twice that of muscle as a whole it is evident that a deduction of a proportionate amount of bicarbonate for the tissue spaces leaves a relatively still smaller bicarbonate concentration for the interior of the fibers and hence a more acid reaction than would be calculated from the bicarbonate content of the whole muscle (see section on pH of muscle).

PERMEABILITY. Like most cells the muscle is permeable to narcotics (Overton, 1902b), lipid solvents, dissolved gases (CO_2, O_2, NH_3, N_2) and

water. It is also permeable to lactate (Conway and Kane, 1934b), urea (P. Eggleton, 1930), creatine (but not phosphocreatine) (P. Eggleton, 1930; Tiegs, 1925), histidine (Eggleton and Eggleton, 1933), and to amino acids (Van Slyke and Meyer, 1914).

The cell has been believed impermeable to sugar, since the muscle shrinks[2] when sugar is added to an isotonic salt solution (Overton, 1902b). Further, neither frog muscles (Overton, 1902b) nor mammalian muscles (Vandervael, 1934) gain in weight in an isotonic sugar solution as they presumably would if sugar could penetrate. When the muscles die in sugar solution they lose weight, showing that they become permeable to their contained electrolytes before they do to sugar. The impermeability of muscle to glucose was directly confirmed by M. G. Eggleton (1935) who soaked muscles in glucose solutions and analyzed subsequently both the muscle and the solution. Glucose penetrated into only 16 to 27 per cent of the muscle (probably only the tissue spaces) but sucrose, strange to say, penetrated the whole muscle without hindrance. It is surprising that no gain in weight was observed under these conditions (cf., however, Bucciardi, 1932). Similar experiments were tried previously by Schulze (1927) who found by chemical analysis that glucose penetrated 36 per cent of the muscle.

Cation permeability. In general the muscle fiber seems to be normally impermeable to anions and to the larger cations, sodium, lithium and calcium, but permeable to potassium, caesium, rubidium, ammonium and hydrogen. The lithium and sodium ions, on the basis of mobility measurements, are believed to be more hydrated and to have, therefore, a larger effective radius (Mond and Amson, 1928). Permeability to rubidium and caesium is also indicated by the work of Mitchell, Wilson and Stanton (1921) and permeability to rubidium by the work of Mendel and Closson (1906). Taubmann (1934) has perfused the legs of frogs with calcium-free solutions and reports no loss of calcium from the muscles, confirming Mond and Amson (1928). Burns (1933) has also shown no change in the calcium content of rat muscles in spite of drastic changes in calcium metabolism, thus indicating impermeability to calcium. There is little evidence available concerning the behavior of magnesium but Wilkins and Cullen (1933) came to the conclusion that magnesium acts in general like potassium and phosphorus in human hearts. Calcium, however, being relatively equal in concentration in muscle and plasma and perhaps also unable to penetrate the

[2] Such evidence is, of course, not altogether reliable since small changes in weight may be due to tissue spaces rather than to cells.

muscle membrane, did not follow the characteristic changes of potassium and magnesium observed in diseased hearts.

Permeability to potassium. Evidence for a permeability of the muscle to potassium comes from the fact that the movements of potassium between muscle and blood or solutions seem to be determined by the relative concentration of potassium and of hydrogen ions inside and outside, as if it were a membrane equilibrium and these were the only penetrating ions. Thus Mond and Amson (1928) and Mond and Netter (1930) showed that in frog muscle perfusions an increase of potassium in the solution above an equilibrium value of 13 mgm. per cent resulted in diffusion of potassium into the muscles and vice versa. Addition of potassium to frog blood plasma in which frog muscles are immersed likewise causes the diffusion of potassium into the muscles against a concentration gradient (Fenn and Cobb, 1935). The diffusion of potassium is also appropriately influenced by the pH according to the expectations of a membrane equilibrium. Thus, if the acidity of the solution is increased, potassium tends to come out or a larger concentration of potassium is required in the solution to give an equilibrium (Fenn and Cobb, 1934; 1935). An increase in acidity inside the cell produced by an increase in carbon dioxide tension and without change in outside pH decreases the loss of potassium. On the other hand, the addition of ammonia to the solution causes rapid loss of potassium because the undissociated ammonia penetrates and raises the internal pH; or possibly the ammonium ion penetrates in exchange for a potassium ion. All these observations can be explained equally well if potassium diffuses as undissociated potassium hydroxide as suggested by Osterhout (1930) instead of by exchanging with a hydrogen ion.

Monauni (1929) found a membrane, composed of the abdominal wall of a frog, impermeable to potassium except for small amounts which he thought diffused through the intercellular spaces when a ten-fold difference in potassium concentration existed between the two sides of the membrane. Since isotonic potassium chloride was used on one side of the membrane, the experiment would be invalidated by the resulting damage to the muscle. Fenn and Cobb (1934) showed that muscles in potassium chloride swell greatly and take up an isotonic solution of potassium, partly as potassium hydroxide, since the chloride taken up is less than the potassium.

Further evidence is available that potassium can diffuse from cells in the mammalian body into the blood where an increase in concentration can be detected. Gershman and Marenzi (1933) showed that in ether

anesthesia potassium left the blood and could not be accounted for in the urine. It presumably went, therefore, into some tissue cells. D'Silva (1933, 1934) and Schwarz (1935) report a transient increase in potassium in the blood after injection of adrenaline, and D'Silva mentions another increase after severe hemorrhage. The loss after hemorrhage was discovered independently by Baetjer (1935) and Thaler (1935). Baetjer added the interesting fact that the potassium escapes from muscles when the blood flow is decreased below a certain critical level. Thaler found further that if the drawn blood, loss of which caused a rise in blood potassium, be then reinjected the blood potassium falls to its normal value, even when the ureters are tied. This seems to indicate that potassium can escape from the cells under conditions of low blood volume or low blood flow and can then diffuse back into the cells again when the blood volume is again increased. This shows that the initial loss of potassium was not due simply to cell destruction which is usually invoked (Peters, 1932) in explanation of an elimination of potassium in excess of the intake.

Anion impermeability. Much of the evidence on this question has already been described in connection with the measurement of tissue spaces. Further evidence will be mentioned in connection with potential measurements on muscle. There seems to be general agreement that the muscle is normally impermeable to chloride. An isolated exception is the work of Winterstein and Hirschberg (1927) who observed that chloride diffused through a membrane made of the abdominal wall of frogs and concluded that muscle is permeable to chloride. It seems probable that the diffusion took place in this case through tissue spaces or injured cells. The same objection can be brought against the experiments of Wesselkina (1932) by the same method in which it was shown that lactic acid could diffuse through such a muscle membrane. Nevertheless it has been shown by Conway and Kane (1934b) that lactic acid can diffuse from a solution surrounding the muscle into all the muscle water, the diffusion constant being, however, distinctly less than that found for diffusion into agar jelly (or into tissue spaces) as shown by Eggleton, Eggleton and Hill (1928). If the lactate diffuses as a negatively charged anion, then this is another exception to the rule of anion impermeability. It is possible, however, that it diffuses as undissociated lactic acid.

Permeability to phosphate. Embden and Adler (1922) first observed systematically the diffusion of phosphate from frog muscle immersed in Ringer solution. They interpreted an increase in phosphate diffusion

as evidence of an increased permeability of the muscle. This phosphate loss is high when the muscle is first immersed, but falls to minimal values within an hour. There is a large increase just before death (Behrendt, 1922). The loss is increased by asphyxia (Simon, 1922; Kitano, 1928; Jljin and Tichalskaja, 1932b) and as a result of subthreshold stimulation (Weiss, 1922). Vogel (1922) found no increase of phosphate loss in potassium chloride contracture, but Fenn (1931) found an increased loss after potassium chloride contracture and after immersion in isotonic sugar solution (confirmed by Cardoso, 1931). The phosphate loss is also increased by stimulation, both direct and indirect (Embden and Adler, 1922; Embden and Lange, 1923; Behrendt, 1922; Weiss, 1922; Wojtczak, 1927a, b; Jljin and Tichalskaja, 1932a; Hykeš and Řeřábek, 1935; Cardoso, 1934).

With the subsequent discovery of phosphocreatine, it became evident that many of these changes could be better explained on the theory that the rate of loss of phosphate was proportional, not to the permeability of the membrane, but to the concentration of free phosphoric acid in the muscle (P. Eggleton, 1929), the membrane being assumed permeable to phosphate. From this point of view, the concentration of phosphate which just prevented phosphate loss from the muscle was determined by Stella (1928) and by Semeonoff (1931). A higher concentration was found necessary for fatigued muscle. There is certainly a good parallelism between the rate of loss of phosphate from muscle and the amount of phosphocreatine breakdown, the latter being increased in isotonic sugar solutions (Cardoso, 1931), in potassium chloride contracture (Hegnauer, Fenn and Cobb, 1934), and, as is well known, in asphyxia and stimulation. Nevertheless, M. G. Eggleton (1933) found that the experiments of Stella and of Semeonoff were complicated by loss of phosphate from bone included with the preparation and concluded that the muscle membrane is normally almost impermeable to phosphate which can diffuse freely into only 20 to 30 per cent of the muscle. The loss, therefore, is perhaps proportional to the amount of free phosphate in the muscle, but is very slow. The question is one of great theoretical importance, for it appears to constitute an exception to the general rule that the muscle is impermeable to anions. It may be possible that the slow loss of phosphate observed is due to the passage of phosphate in some unionized organic combination. The diffusion of some such compounds has been studied by Rothschild (1929). This suggestion does not help much, however, unless it can be shown also why these compounds do not diffuse out of the resting muscle, while they can diffuse during activity.

In the resting muscle, therefore, the inorganic phosphate may be very low, but there is evidence from isolated muscles that this amount may come into equilibrium, though slowly, with the phosphate outside. Even a slow leak of this sort, however, is difficult to reconcile with the simple membrane theory, for then potassium phosphate could slowly escape. It is possible that in the body the membrane of the resting muscle, at least, is quite impermeable to phosphate, but it seems more likely that the theory is fundamentally inadequate in its present form to cover all contingencies.

EVIDENCE CONCERNING PERMEABILITY FROM SALT POTENTIALS. If a muscle is in contact with two different solutions at the same time and the potential difference between these two solutions is measured, the resulting salt potentials give some indication of the relative mobilities of the different ions in the muscle substance. In practice, one of the solutions is Ringer's solution which is kept constant, the other being varied. It is known from the work of Höber (1905), Seo (1924) and Sugi (1934b) that a change in the anion has relatively little effect upon the potential, while a change in the cation, particularly the potassium ion, has a large effect. Thus Seo (1924) found that potassium chloride caused a negative potential of 38.9 m.v. while sodium chloride caused a positive potential of 14.9 m.v., a total variation of 53.8 m.v. Among the different sodium salts, however, the extremes observed were a positive effect of 11.5 m.v. in the iodide and a positive effect of 3.2 m.v. in the sulfate, a range of only 8.3 m.v. From these results it may be concluded that the muscle is more permeable to cations than to anions. The small anion effect which does appear may indicate a slight permeability to anions, but the results may also be complicated by variable short circuits in the tissue spaces or extracellular potentials.

Oker-Blom (1901) found that distilled water caused a marked positive effect. This was confirmed by Sugi (1934b) and Fenn (1931) found that isotonic sugar solution caused a similar large positive potential (30–40 m.v.). In this case the diffusion potential depends entirely upon the ions which are diffusing out and the positive potential may perhaps be taken to indicate that the positive ions again have a higher mobility in the muscle substance than the negative ions. An initial transient negativity is found with both water and sugar, which may be due, as Oker-Blom suggested, to the greater mobility of chloride than sodium as they diffuse out of the extracellular spaces.

Du Bois-Reymond, who discovered the injury potential of muscle (see Oker-Blom, 1901) explained it on his "molecular" or preëxistence

theory according to which the injury simply uncovered a potential difference which previously existed on the normal surfaces. This theory was better formulated by Bernstein (1902) with his "membrane" hypothesis, according to which the normal cell is polarized so that it is positive outside and negative inside, this potential difference being determined by the concentrations of potassium. A measurement of the injury potential on this theory should give a value which agrees in general order of magnitude with that calculated from the formula 58 log K_i/K_o m.v., it being assumed that the mobility of the anions is zero. Hegnauer, Fenn and Cobb (1934) found values which were only a little more than half the expected value. Since this difference can be explained by shortcircuiting in the tissue spaces and by possible incomplete elimination of potential at the injured end, it might be concluded that measurements of the injury potential confirmed as well as could be expected the theory that the muscle is surrounded by a membrane permeable only to potassium.

Some further explanation is necessary, however, for the positivity due to the application of water or isotonic sugar to muscle. It cannot be due to the higher concentration of potassium inside in combination with anions of low mobility, for this condition obtains also in Ringer's solution before the water is applied. It might be supposed that a part of this potassium potential was balanced in Ringer's solution by an opposite potential due to sodium chloride, the sodium having a higher mobility in the membrane than the chloride. When sodium chloride is replaced by water the full potential due to potassium is then revealed. This appears a priori as the most probable explanation but it does not fit well with chemical evidence indicating impermeability to sodium. Another possibility is that the loss of sodium chloride diffusing from the tissue spaces into the water removes the extracellular short circuit and so increases the measured potential. Actually the sum of the injury potential and the positivity increase caused by water is nearly equal to the theoretical potential calculated from the difference in concentration of potassium between the inside and the outside of the cell. It is difficult, however, to see how this short circuit could be so effective, especially when the other end of the muscle is uninjured in Ringer's solution. The only other possible explanation seems to be that the water or sugar modifies the muscle membrane or the muscle substance in such a way as to increase the mobility of the cations as compared to the anions. In any event these positive water potentials have not been sufficiently taken into consideration in the past. If they really

mean the elimination of a sodium potential (with negativity outside) they indicate the need for important modifications of existent theories.

The "preëxistence" theory represents the most commonly accepted interpretation of salt and injury potentials but there is a considerable difference of opinion. Höber (1905) himself was impressed by the fact that anions are not without some effect and felt that Bernstein's membrane hypothesis was not confirmed by his work. Steinbach (1933) believed that the injury potential is largely due to processes occurring at the point of injury, this opinion being largely based upon the fact that in the scallop muscles which he was using the application of potassium chloride to the longitudinal surface did not cause negativity as it does in frog muscles and as it does even in scallop muscles on injured surfaces which have had time to "recover." The recovery of the injury potential which he observed, particularly under the influence of calcium, is not unusual. Sugi (1934a) also supported the original "alteration theory" of Hermann (see Oker-Blom) as a result of a study of the distribution of potential around an injured muscle immersed in a large dish of water. According to him the results could be explained by the supposition that in injury a disc of tissue was added near the injured end, polarized plus inside and minus outside. He did not observe, however, that exactly the same result would be produced by the removal from the same point of a disc polarized in the opposite sense (Wilson et al., 1932). The physical systems which Sugi studied as models of an injured muscle must have been inadequate for some reason.

Szabuniewicz (1930) has shown how extremely sensitive the surface of frog muscle is to potential change caused by injurious contact with the outside of the skin. According to the membrane theory this should also modify the functional behavior of the muscle. Indeed, simultaneous measurements of the injury potential and irritability (rheobase) led Okabé (1934) in a series of articles to the conclusion that rheobase varies inversely with the injury potential. Voelkel (1921), however, came to the opposite conclusion. Some theoretical discrepancies of this sort were pointed out further by Hegnauer, Fenn and Cobb (1934).

The nature of the muscle membrane has been studied by the salt potential method and the general conclusion has been reached that it is not of the nature of an oily layer immiscible in water (Höber, 1910; Vorschütz, 1921a, b; Natannsen, 1922; Matsuo, 1923. Cf. also Mond, 1924; Netter, 1928.)

The effect of a ten-fold change in concentration of salts, particularly of potassium chloride, on the salt potential can be estimated from data

of Fischer (1924) and Sugi (1934a, b). Both indicate about 25 m.v. instead of the 58 m.v. expected theoretically at 20°C. if the membrane is completely anion impermeable. This may be explained partly by an inert extracellular short circuit and partly by neglect of the mobility of the anions. Steinbach (1935) has included anionic mobilities in calculating his expected diffusion potentials. A somewhat similar formulation by Teorell (1935) seems promising. For a theoretical treatment of these salt potentials see Michaelis (1926, p. 209).

In general the salt potentials seem to confirm the membrane hypothesis rather better than Höber (1905) originally believed, but further refinements in the theory are to be expected.

POTASSIUM AND SODIUM. *Amounts in normal muscles.* Since these two bases together comprise nearly all the total base in muscle it is generally true that a high potassium content indicates a low sodium content and vice versa. Reference will be made therefore chiefly to the potassium content but figures for sodium are included in many of the papers referred to.

The earliest analyses of *skeletal muscle* are those of Katz (1896) who found a low value, 240 mgm. per cent potassium per 100 grams of fresh muscle, in the eel, and a high value of 465 mgm. per cent in the hen muscles. Muscles of twelve other animals gave intermediate values, the muscles of man having a fairly average content of 320 mgm. per cent. Similar values have been obtained by later investigators, notably Costantino (1911), Lematte, Boinot and Kohane (1928), Boutiron and Genaud (1928), and Norn (1929). In various muscles of invertebrates Bernard (1931) gives a high value of 623 mgm. per cent potassium in the mantle of *Sepia officinalis* and a low value of 312 in the foot of *Helix pomatia*. According to Leulier, Bernard and Béruard (1933) the potassium content in skeletal muscles of the dog and the pigeon increases with age. In animals which are inactive at birth the muscle potassium is low and increases with age as the water decreases (Leulier and Bernard, 1934). In frog muscles, Fenn and Cobb (1934) found an average potassium content of 330 mgm. per cent and Mitchell and Wilson (1921) 338 mgm. per cent, the values in individual animals kept under different conditions and at different seasons of the year being subject to large variations (cf. Gradinesco and Degan, 1935).

Cardiac muscle contains normally less potassium than skeletal muscle (Leulier, Velluz and Griffon, 1928; Kochmann, 1907; Wilkins, 1934) but the reverse may be true in pathological cases (Cullen, Wilkins and Harrison, 1933). The right ventricle contains slightly less than the more

heavily worked left ventricle (Calhoun, Cullen, Clarke and Harrison, 1930; Cullen, Wilkins and Harrison, 1933; Wilkins, 1934).

Smooth muscle usually contains less potassium than either heart or skeletal muscle (Wilkins, 1934; Leulier, Velluz and Griffon, 1928). The uterus is particularly low in potassium (Costantino, 1911), a value of 98 mgm. per cent having been recorded by Wilkins (1934). Stomach and bladder may not be any lower, however, than skeletal muscle (Costantino, 1911; Wilkins, 1934). Saiki (1908) analyzed stomach and bladder muscle of the pig and suggested that these muscles differed from striated muscle chiefly in the greater amount of extracellular material, the chloride space being 47 per cent of the whole muscle, instead of 13.2 per cent as in striated muscle. Meigs (1912), however, does not find the tissue spaces especially large in frog stomach and suggests rather that smooth muscle lacks the semipermeable membranes of skeletal muscles as shown by its very different weight changes during immersion in experimental solutions. In general skeletal muscle is relatively richer in potassium than smooth muscle but this is quite variable, and Lematte, Boinot and Kahane (1928) were unable to formulate any rule in the matter.

Ernst (1934) has made the interesting statement that the potassium recovered from muscle tissue contains an unusually large fraction of the radioactive isotope of potassium of molecular weight 41, but the experimental basis for this has not yet been published.

Location of potassium. In tissues which contain as much potassium and as little interstitial fluid as muscles it is quite obvious that the potassium is located chiefly inside the cells. Histological microchemical methods have located the potassium on the surface of the cells (Bureau, 1934) or inside in the dark discs (Macallum, 1905; Woerdeman, 1924) but such conclusions are always subject to doubt and since potassium is almost the only cation inside and since it is all (or nearly all) necessary to make the osmotic pressure equal inside and out, it is difficult to suppose that the distribution of potassium in the normal untreated cell is essentially different from the distribution of the water. This objection also applies to the finding of Scott (1932) by the micro-incineration technique that all the ash of striated muscle fibers is in bands corresponding to the anisotropic layers. This would mean that regions containing practically distilled water were adjacent within the same muscle fiber to regions containing a potassium solution of nearly double isotonic strength. It seems not unlikely that the salts could be redistributed over microscopic distances at some time during the processes of fixation, sectioning and incineration.

Reference to table 1 will show that if all the 82.6 millimols of potassium is evenly distributed in all the 660 cc. of water assigned to the interior of the fibers it is present in a concentration of 125 millimols per kilo while the concentration of potassium in an isotonic potassium chloride solution (0.9 per cent) is 120 millimols per kilo.

Bound potassium. Neuschlosz, in a series of articles (1923, 1924, 1926; also Neuschlosz and Trelles, 1924) has insisted that a certain fraction of the potassium in muscle cannot be extracted by isotonic sodium chloride from minced muscle, and has even maintained that denervation increased the amount of potassium bound in this way. If some of the potassium is combined with relatively indiffusible protein as an anion it is not surprising that some of the potassium should be retained as long as the fragments of muscle are immersed in water. If immersed in saline the potassium would presumably escape in proportion to the penetration of the sodium. At any rate other investigators are able to extract all the potassium by saline solutions (Raab, 1927; Sager and Roth, 1930; Sager, Roth, and Kreindler, 1930; Damboviceanu, Sager and Roth, 1930; Callison, 1931). Stanton (1923), Mitchell (1928) and Ernst and Takacs (1931) found, however, that all the potassium could not be removed from frog muscle by prolonged perfusion. All of these procedures, however, are so drastic that they tell little about the condition of potassium in normal muscle. For reasons already mentioned, indiffusibility of part of the potassium does not mean, necessarily, that all the potassium is not free to exert an osmotic pressure.

Ernst (1931) and Ernst and Fricker (1934a) have pointed out that if some of the water in muscle is bound water this would leave room for some bound potassium. Moran, however, (1930) measured the amount of water turned to ice at different temperatures as indicated by the increase in volume and concluded from his own data that there is little if any bound water in muscle. Furthermore, J. Brooks (1934) weighed muscles after equilibrating them at different vapor pressures and showed that there is not over 6 per cent of bound water in muscle. Hill (1930) by a thermoelectric vapor pressure method concluded that frog muscle contains 77 per cent free water (80 per cent total). Using this figure, the concentrations of muscle electrolytes (table 1) may be expressed in mols per liter of free water; the concentration then becomes 257 millimols per kilo in the muscle as compared to 240 in the plasma. It remains, therefore, possible on this basis that a small fraction of the potassium present in muscle is combined in some form which prevents it from exerting an osmotic pressure.

In blood there has been some indication that some of the potassium is bound (Greene, et al., 1931a, b; Rona and Petow, 1923). This conclusion depended upon the finding that the concentration of potassium in an ultrafiltrate of blood was less than the concentration of potassium in the plasma. The facts were confirmed by Ingraham, Lombard and Visscher (1932), but were interpreted rather as a decreased activity of the potassium ion in the blood. A similar decreased activity of potassium inside the muscle fiber is likewise to be expected and is perhaps a better interpretation of a small amount of bound potassium.

Sodium in muscle. The sodium content of muscle is almost always slightly in excess of the chloride content (if both are expressed in equivalents). In human plasma, the Na/Cl ratio is 1.34 (Heubner, 1931) while in muscle it is 1.76 (Katz, 1896). In frog plasma and frog muscle the Na/Cl ratios are, respectively, 1.47 and 2.14 (Fenn, Cobb and Marsh, 1934). If the interstitial fluid in muscle approximates in composition an ultrafiltrate of serum, then it should have a lower Na/Cl ratio than the plasma (Ingraham, Lombard and Visscher, 1932; Sjollema and Seekles, 1933), the decrease being chiefly dependent upon the pH. Since in muscle the Na/Cl ratio is greater than in plasma it is evident that some of the sodium must be elsewhere than with the chloride in the interstitial fluid. This was confirmed in frog muscle by Fenn, Cobb and Marsh (1934) by finding that some of the sodium, about equal to the amount present in excess of the chloride, did not diffuse out into isotonic sugar solution along with the chloride. Comparing the sodium and chloride contents of frog muscle and plasma and expressing the former in percentage of the latter, i.e., calculating the sodium and chloride spaces of muscle, it is found that the sodium space is 24.5 per cent of the muscle and the chloride space is only 14.5 per cent. On soaking in Ringer's solution, the sodium space increased to 33 per cent and the chloride space to 31 per cent so that the Na/Cl ratio became 1.06. These results are consistent with the view that during soaking the muscle fibers gave up some water to the tissue spaces and the space originally containing sodium but not chloride became permeable to chloride also. Mond and Netter (1932) have studied particularly the amount of excess sodium (over chloride). They mention some seasonal changes in the amount of excess sodium in frogs and believe that the amount is dependent in some way upon the magnitude of the oxidative quotient of Meyerhof which obtains during recovery from stimulation. Mond and Netter consider this excess sodium to be combined in some way in the surfaces of the muscle fibers, but there seems to be no reason why it could not

be inside the fibers along with the potassium, but unable to penetrate the membrane.

Most of the sodium interchanges between tissues and blood can be explained by the theory that tissue sodium is all extracellular. There are cases, however, where sodium without chloride appears to enter the cells in exchange for potassium, sometimes because the membrane is damaged and sometimes apparently without injury, as in stimulation. Wu and Yang (1931) injected 25 per cent sodium chloride intrapleurally in rabbits and observed that the total base of the muscles increased six times as much as the chloride. They concluded that muscle is normally permeable to sodium. It is unfortunate that they did not analyze directly for sodium.

POTASSIUM VS. SODIUM CHLORIDE IN MUSCLES. Since potassium supplies the chief cation equivalents of the muscle fibers, two factors may be said to determine the potassium content of muscles: 1, the osmotic pressure or total base of the blood; and 2, the amount of intact muscle fiber in the whole muscle. Perhaps a further subdivision will be possible if it can be shown that potassium is more concentrated in the muscle fibrils than in the sarcoplasm, but concerning this possibility we have no sure basis at present. Since the osmotic pressure is fairly constant in different animals and in the same animal under different conditions the chief variations in potassium content of muscles must be due to the volume of muscle fibers present. Since further the total base of muscle must be fairly constant a decrease of potassium must be accompanied by an increase in sodium and in chloride.

This reciprocal behavior of potassium and sodium is well illustrated in the electrolyte changes which go on between frog muscle and the Ringer's solution in which it is immersed. Thus Fenn (1935) found a loss of 1.63 m.-eq. per cent of potassium, 0.23 m.-eq. per cent of magnesium and 0.2 m.-eq. per cent of bicarbonate, and a gain of 1.3 m.-eq. per cent sodium and 1.7 m.-eq. per cent chloride. In general, sodium chloride comes in and potassium goes out. The net result of these known changes is a gain of 2 m.-eq. per cent of acid which is partly balanced by phosphocreatine changes but which leaves an excess of base in the Ringer's solution.

Similarly, Mauriac, Aubel and Boutiron (1927) found a loss of potassium and a gain of sodium and chloride by rabbit muscles immersed in edema fluid. According to Genaud (1928) this also occurs not only in physiological solutions but also in serum. In both cases the gain in sodium exceeds the gain in chloride. In human muscles in edema the

Na/Cl ratio was also found to be higher than normal (Blum and Broun, 1927). A similar loss of potassium and gain of sodium chloride goes on in vivo in experimental nephritis in rabbits (Nau, 1928) and in guinea pigs raised on a vitamin C-free diet (Randoin and Michaux, 1932). It was shown later by Michaux (1934) that in such acutely scorbutic muscles the magnesium was decreased and the calcium increased.

Denervation of rat muscle results in a loss of potassium which is parallel to the increase in non-muscular tissue (Hines and Knowlton, 1933) and presumably parallel to the increase in sodium and which is accompanied by an increasing chronaxie (Leulier et al., 1932 (2), cf. also 1935). Wilkins and Cullen (1933) have found the K/Na ratio greater in normal than in diseased human hearts. They conclude that potassium, phosphate and magnesium decrease together while sodium increases. This work confirmed the findings of Calhoun, Cullen and Clarke (1930) and of Harrison, Pilcher and Ewing (1930). Similar changes occur in stimulation as will be mentioned later.

pH OF MUSCLE. As already indicated, the theory that the potassium content is controlled by a membrane equilibrium demands a low pH inside the cell. Yet the majority of workers have concluded that the pH of muscle is slightly alkaline. It seems possible, however, that this conclusion is based upon faulty evidence. Thus all the electrometric methods using manganese dioxide, glass, quinhydrone and hydrogen electrodes (Ritchie, 1922; Meyerhof and Lohmann, 1926; Furusawa and Kerridge, 1927; Mackler, Olmsted and Simpson, 1930; Voegtlin et al., 1934) yield values for the pH between 7.0 and 7.8 but it seems unlikely that these electrodes could come into equilibrium with the inside of the cells and the result obtained may be rather the pH of the outside solution with which the inside is in equilibrium. In some of these cases, extracts of muscles were measured and it seems possible that the anion-impermeable cells may permit the extraction of more base than acid.

With the use of phenol red, Margaria (1932) reports a pH of 7.48 which changes to 6.07 on stimulation. Later (1934) using bromcresol purple or bromthymol blue he observed that the pH became apparently more alkaline on stretching the muscle, a result which he felt was probably attributable to some optical artifact or to some change in the physicochemical properties of the muscle proteins under stress. Rous (1925) found quite acid reactions inside mouse muscles but there is ground for suspicion that his muscles were injured in the technique of observing the color. When dyes are simply injected into the animal or when the muscle is immersed in a solution of the dye, it is difficult to be sure that

the dye is really inside the cell and not in the interspaces. At all events, when dyes are actually injected into the interior of individual cells, values between 6.6 and 6.9 have been obtained (Schmidtmann, 1924; 1925; Chambers, Pollack and Hiller, 1927).

The surest basis for estimations of the pH inside muscles seems to be the determination of the carbon dioxide combined at known carbon dioxide tensions. The validity of this calculation and the choice of pK_1 used in the Henderson-Hasselbalch equation, has been discussed by Fenn and Maurer (1935). The chief uncertainties of the method would tend to make the pH too high rather than too low. It is concluded that in frog muscle the pH is about 6.9 inside the fiber. This agrees with the figures of table 1 which show a lower bicarbonate content in the muscle fibers than in the plasma.

The carbon dioxide dissociation curve of frog muscle was measured by Fenn (1928), Stella (1929) and Root (1933). Similar curves have been obtained by Brody (1930) in heart muscle, by Irving, Foster and Ferguson (1932) in mammalian muscle, and by Cowan in crab muscle (1933). Fenn and Maurer (1935) have called attention to a striking change in the bicarbonate equilibrium which occurs after dissection of frog muscles. Within the body, as a result of the greater acidity of the interior of the muscle, the bicarbonate concentration outside the cells is 2.6 times that inside, while after dissection and immersion in Ringer's solution, the outside to inside bicarbonate ratio becomes 1.0 (Fenn and Cobb, 1934), or 1.2 (Brookens, 1933), or 1.15 (Fenn and Maurer, 1935). The difference in pH between the inside and outside of the fiber is less after immersion in Ringer than that normally existing in vivo. Even if the dissected muscle is immersed in blood of the same animal this change occurs and the bicarbonate content of the muscle steadily increases, coincident probably with the enlargement of the tissue spaces.

Netter (1934) has also concluded that the pH is acid inside the fibers, his conclusion being based in part on the amount of ammonia taken up by the cells in comparison to that outside.

This acidity inside the fibers is, however, not sufficient to agree with the high potassium concentration inside on the membrane-equilibrium theory, according to which H_i/H_o should equal K_i/K_o. Some modification of the theory is required to meet this objection. Perhaps the lactic acid and phosphocreatine buffer mechanisms keep the pH too alkaline in spite of the demands of the membrane equilibrium. It is also possible as already suggested that the membrane equilibrium may apply only to some space inside the fibers such as the fibrils or the anisotropic discs within which the pH may have the required value.

It is impossible within the limits of this review, to consider all the changes of muscle electrolytes which occur in the body as a result of acid-base changes in the blood. It must suffice to outline the behavior expected on the basis of the physico-chemical organization of the muscle as it has been presented here. The red blood corpuscles are better buffered than the plasma, and the plasma (plus corpuscles or true plasma) is better buffered than the muscles. Consequently an increase of carbon dioxide tension in the body would produce the greatest increase in acidity in the muscles. To equalize this change, acid would tend to diffuse from the muscles toward the corpuscles and base in the reverse direction. The red corpuscles, however, are impermeable to base and the muscles to acid. Hence the changes which may be expected to occur are a diffusion of acid into the corpuscles (the chloride shift) and a diffusion of base into the muscles (the potassium shift) (Fenn and Cobb, 1935). This potassium shift has only been observed, however, in frog muscles, and according to Gollwitzer-Meier (1925) it does not occur in man. It is not surprising that this should be the case for the increase in acidity might be compensated for more quickly by a breakdown of phosphocreatine or a diminution in the rate of formation of lactic acid in muscle, than by a diffusion of potassium. Thus, for example, Haldi (1933) has shown that the lactic acid content of muscles increases when sodium bicarbonate is injected intravenously.

When carbonic acid is added to frog muscle immersed in frog blood potassium is driven from the blood into the muscle to neutralize the acid inside the muscle. When hydrochloric acid is added, which cannot penetrate the membrane, it might be expected that potassium would have to diffuse out of the cells into the blood to neutralize the acid. Actually, however, it still goes into the cells if sufficient acid is added to have any effect at all (unpublished observations). This constitutes an exception to the theory unless it can be shown that some other acid has penetrated or that under these conditions the muscle has been stimulated, perhaps to form lactic acid. Banus, Katz and Mull (1929) have done a somewhat analogous experiment in perfusing dog muscle with hydrochloric acid added to the perfusate. In passing through the muscle this increased acidity was partially neutralized. In the absence of a more complete study of the electrolytes it is impossible to be satisfied with the conclusion of the authors that hydrochloric acid simply diffused into the muscles. No increase in calcium was found but possibly some potassium diffused out. According to Linder (1927) hydrochloric acid administered to man is partly excreted in combination with potassium from the cells.

MUSCLES IN ACTIVITY. *Electrolyte changes.* Many investigations show that under certain conditions muscles lose potassium and gain water, sodium and chloride on stimulation. This is an important fact to be reconciled with any theory of muscle electrolytes.

Mitchell and Wilson (1921) stimulated one leg of a perfused frog and found no increased loss of potassium except when the muscle was stimulated to exhaustion. Mitchell, Wilson and Stanton (1921) repeated the experiment in frogs perfused with a solution containing rubidium. After washing out the rubidium solution from the vessels by perfusion with sugar they analyzed the muscles for rubidium and found small amounts present in the stimulated muscle. They concluded that the rubidium which entered the muscle in exchange for potassium had been rendered indiffusible by stimulation and inferred that the same thing happens to potassium in a normal muscle. Ernst and Scheffer (1928) showed similarly that stimulation during perfusion of frog gastrocnemius muscles results in a loss of 30 to 50 per cent of the potassium, extreme stimulation being unnecessary. Ernst and Csúcs (1929) showed that sodium and chloride were gained and potassium and phosphate were lost as a result of direct stimulation of the perfused muscles but no such changes were observed in indirect stimulation. Later Ernst and Fricker (1934b) found a loss of potassium by indirect stimulation only when the muscles were perfused with Ringer's solution containing high potassium concentration and low calcium which was supposed to render them more permeable. With 57 mgm. per cent potassium in the perfusate Netter (1934) found that the muscles of frogs continued to take up potassium even when stimulated. Mond and Netter (1930) also found on perfusion of frog muscles with Ringer containing 13 mgm. per cent potassium (which normally is in potassium equilibrium with frog muscle) that indirect stimulation caused no loss of potassium or gain of chloride which could be detected in analysis of the perfusate. The conclusion to be drawn from these results is that under the most physiological conditions stimulation may occur without loss of potassium or gain in sodium and chloride. Ernst would conclude also that stimulation results in a breakdown of potassium compounds in the muscle and an increase in the concentration of diffusible potassium which results in a loss of potassium only when the permeability is also increased. This, however, is an interpretation which is hardly required by the facts. All these experiments suffer from the fact that the muscles do not retain their normal blood supply during the experiment. Fenn and Cobb (1936) have found, however, that even in frog muscles in situ with in-

tact circulation a loss of potassium on stimulation could only be shown with certainty when the muscle was stimulated directly, thus confirming in general the previous work on frogs. It is possible, however, that frogs are exceptional in this respect.

Embden and Lange (1923) reported an increased uptake of chloride by muscle as a result of stimulation, but their evidence was not sufficiently direct to be convincing. Fenn (1935) has shown, however, that on stimulation of frog sartorius muscles in Ringer's solution the muscle takes up some of the solution together with its contained chloride (presumably in the tissue spaces) together with some pure water for the interior of the fibers. There is, therefore, an uptake of chloride but no evidence that it is taken into the interior of the fibers. This is true also in stimulated rat muscle.

A loss of phosphate on stimulation, both direct and indirect, of frog muscle in Ringer's solution has already been described.

Conflicting reports are available concerning electrolyte changes during activity of human muscles. Dill, Talbot and Edwards (1930) summarize the findings for non-fatigued subjects by the statement that fluids containing diffusible ions move from the blood to the muscle, there being in addition an increase in phosphate and calcium in the plasma and an increase in lactate balanced by a decrease in bicarbonate. This increased ultra-filtration of plasma may be the result of an increased capillary pressure during exercise and would explain an increased water content and chloride content of the muscles which is found during stimulation. Christy (1927) has also found a decrease of chloride in the blood in exercise which may be similarly explained. The fact that sodium chloride also increases in isolated muscles on stimulation in Ringer's solution (Fenn, 1935) indicates, however, that increase in capillary pressure may not be the only explanation of the phenomenon.

In greater degrees of fatigue in man more extensive electrolyte changes are to be expected. Ewig and Wiener (1928) find that potassium and phosphate increase in the blood while calcium and sodium decrease. The sodium leaves the blood partly as sodium chloride and partly as sodium bicarbonate. An increase of potassium in blood was also found by Schenck (1925) while Tómasson (1928) found conflicting results in three cases and concluded that there was no significant change.

Behrendt (1925) found no changes in blood calcium or potassium after tetanus toxins or fatigue from running, in dogs. Zagami (1928) found an increase in both calcium and potassium in the blood of certain dogs as a result of stimulation of the motor centers in the cortex. Wacker

(1929) stimulated the muscles of rabbits under urethane anesthesia and found a 50 per cent increase in calcium in the blood and a 140 per cent increase in magnesium.

After exercise in trained rabbit muscles, Fomin (1930) has found a decrease of muscle potassium lasting 1 to 2 days followed by an increase lasting 4 more days. In dogs only an increase was observed. In epileptic convulsions, McQuarrie (1932) has found an increased excretion of potassium due to loss from cells and Hirschfelder and Haury (1935) have found increased potassium or decreased magnesium in the blood of man. Baetjer (1935) has observed an increase in potassium in the blood after exhausting stimulation in cats and Kehar and Hooker (1935) have found a loss of potassium from the heart during ventricular fibrillation. Beznák (1931) reported an increased concentration of calcium and of phosphate in serum after strychnine convulsions. Likewise Goldberg, et al. (1931) found calcium, phosphate and also potassium in the serum increased after severe work in dogs and rabbits. In sleep the potassium and calcium of the plasma in dogs are decreased while the reverse is true in conditions of general motor or psychic stimulation by drugs (Cloetta, Fischer and van der Loeff, 1934). Potassium and calcium are also increased in the blood of rabbits after stimulation of the muscles according to the same authors. The muscles were also analyzed and showed diminished calcium and potassium contents, but no account was taken of the change in dry weight of the tissue. The increased water content would just about explain their observations except the fact that the percentage loss of calcium exceeded that of potassium. It must, of course, be recognized that electrolyte changes in muscle can be established with certainty only when the content of the dried muscles is known. Changes observed in the blood also are subject to a variety of interpretations and can be accepted as evidence of gain or loss from muscles only with due reservations.

If muscles are impermeable or only very slightly permeable to phosphate, as claimed by M. G. Eggleton (1933), then no loss or only a small loss from active muscle is to be expected in spite of an increase in phosphocreatine breakdown. Some such increase in blood phosphate in exercise has already been mentioned. Such an increase was reported also by Havard and Reay (1926), but Gemmill and Ribeiro (1933) found no change in man after short, violent exercise except for a slight increase in acid-soluble organic phosphate, and in dogs only a small increase after prolonged exercise. Likewise, Houget (1933) found that the total phosphorus content of dog muscles remains constant even after

prolonged activity and this agrees with unpublished experiments on rats by the author.

With the exception of Ewig and Wiener (1928) most of the workers quoted above who have studied calcium changes on stimulation have agreed that it increases in concentration in the blood or decreases in the muscles. In addition, Weise (1934) showed that after stimulation of rat muscles there was an increase in diffusible calcium in the muscle although no change in total muscle calcium or in blood calcium was observed. This increased diffusibility was apparently not due simply to an increased acidity.

After 30-minute indirect stimulation of muscles of anesthetized rats, Fenn and Cobb (1936) have found electrolyte changes (per kilo dry weight of muscle) which they interpret as 1, a loss of potassium from the cells in exchange for sodium; 2, an increase in the intercellular spaces resulting from the addition of an ultrafiltrate of plasma (water + sodium chloride), and 3, a further intake of water by the cells due to an increase in the concentration of osmotically active constituents in the muscle. Since these changes were largely reversed in 2 hours of recovery they were not due to irreversible injury to the cells.

The increase in extracellular fluid might be caused by the increased capillary pressure. The exchange of potassium for sodium would result from an increased permeability of the membrane such that sodium was admitted but not chloride. The process of recovery is then difficult to explain, however, since the membrane, having regained its normal impermeability, would prevent the loss of the sodium which the muscle had gained. Some rather artificial hypotheses were advanced in an attempt to reconcile this finding with the membrane theory of potassium permeability. The difficulty suggests, however, that a better or more complete theory is the real need.

Muscle pH in activity. An acidification of the muscles on stimulation has been noted by most authors who have tried to measure the pH of muscle. (Ritchie, 1922; Furusawa and Kerridge, 1927; Margaria, 1932; Voegtlin et al., 1934; Pechstein, 1914; Schade et al., 1921; Meyerhof and Lohmann, 1926; Fenn, 1928; and Wetochin, 1926.) In addition, mention may be made of the work of von Studnitz (1934), who noted that acidification occurs first in the anisotropic discs but is absent in muscles poisoned with iodoacetate. In the early stages of anaerobic fatigue under normal carbon dioxide pressures no change in pH, or even a slight alkalinity, is to be expected from the work of Lippmann and Meyerhof (1930). Under higher carbon dioxide pressures the alkalinity may be

of considerable magnitude. In the later stages of fatigue, when measurements have usually been made, lactic acid formation always exceeds phosphocreatine breakdown so that the muscle becomes acid.

Margaria (1934) has noted, by an indicator method, a sudden apparent alkalinity on stimulation, which was so large and so prompt that he attributed it to some artifact due to the tension development rather than to true chemical change.

Water content in activity. It has been known since the work of Ranke (1865) that stimulation results in an increased uptake of water by muscles. Fletcher (1904) showed that this was true for isolated frog muscles only as a first effect of stimulation since later, after some recovery, the stimulated muscle may begin to lose water to such an extent that it may contain less water than the resting control. A similar gain in weight was found by Back, Cogan and Towers (1914) in frog muscles stimulated in vivo, and by Barcroft and Kato (1915) in dogs. The result is due to an increased osmotic pressure inside the fibers. Hill and Kupalov (1930) found in extreme fatigue an increase in vapor pressure of the muscle corresponding to a change from 0.731 per cent sodium chloride solution to a 1.08 per cent solution. Known chemical changes sufficed to explain an increase to 0.906 per cent sodium chloride. Likewise Meyerhof (1930) found that the freezing point depression was increased in fatigue about 30 per cent, of which only three-fourths could be explained by transformation of phosphate, lactic acid, and ammonia. These facts explain well an intake of water by muscles during activity. It is interesting to note that no such intake of water was observed in man during exercise by Dill, Talbot and Edwards (1930) nor by Ewig and Wiener (1928) in more exhausting work. Possibly this failure (to observe an uptake of water by muscles in excess of that associated with the chloride which disappeared) may be due to lack of excess available water in the body.

Protein. Ørskov (1932) has found a 15 to 20 per cent increase in the swelling of muscle proteins after activity. The swelling was estimated from the volume of the sediment obtained by centrifuging from a 0.1 N sulfuric acid solution a muscle brei obtained by pulverizing frozen muscles. The result is not inconsistent with the finding of Hensay (1930) that stimulation increases the solubility of muscle proteins in acid solutions although in alkaline solutions (pH 7.2) the reverse is true as Deuticke (1930) also showed. Deuticke (1932) found further that this decrease in solubility is proportional to the amount of tension developed and is reversible in oxygen but not in nitrogen.

Changes in the proteins of muscle on stimulation are also attested by the changes in opacity and in double refraction which have been observed by von Muralt (1932, 1933, 1934) and by von Baeyer and von Muralt (1933). The correlation between these various protein changes is not altogether clear.

Changes in conductivity in activity. For an understanding of electrolyte changes in stimulation pertinent information may be obtained from measurements of changes of electrical conductivity at different frequencies of alternating current. In this way Achelis (1932) found evidence for a decreased permeability of the muscle membranes during activity. Dubuisson (1934) on the other hand finds an increased permeability (decreased ohmic resistance and increased polarization capacity) in two waves coinciding with the spike of the action potential and the after potential. Bozler (1935) attributes these differences to the types of electrodes used and to the thickness of the gastrocnemius muscle, and reports with improved technique an increase of 5 to 10 per cent in the resistance (at 1000 to 10000 A.C. cycles) during a maximum isometric twitch of the frog sartorius muscle. Earlier papers on this subject are quoted by Bozler. In a further more detailed study of muscles in rigor as an example of a stimulated muscle, Bozler and Cole (1935) found no change in the internal conductivity of the fibers at infinite frequency, indicating no liberation of previously bound potassium and no change in the phase angle of the membrane, indicating, as they suggest, no change in its selective permeability on stimulation. The increase in resistance observed by Bozler seems to be due, therefore, to an increase in the resistance of the membrane to low frequencies. It may be mentioned that the conclusion is dependent upon the assumption that the tissue spaces do not decrease in volume during stimulation.

In these experiments there is little evidence for a sudden breakdown of the muscle membrane which would permit the loss of potassium and give rise to the action current. Even the decrease in resistance found by Dubuisson (1935) during the time of the action current amounts to a change of only 2 per cent, which is hardly sufficient to account for the action current or for the loss of potassium. On the other hand, Blinks has found a marked decrease in resistance associated with the action current in Nitella (personal communication from Dr. Osterhout). In muscle it remains doubtful whether the membrane which limits the escape of potassium is identical with the membrane which mediates the action current. The fact that the contractile mechanism can be stimulated without arousing an action current (Gelfan and Bishop, 1933) suggests

likewise an independence of the action-current mechanism. The electrolyte changes in activity may then be a result of the contractile process rather than of the action-current process.

CONCLUSIONS

This review has been presented chiefly from the point of view of the theory that the muscle membrane is impermeable to all common ions except the potassium and the hydrogen ion. This theory explains satisfactorily most of the facts. There is, however, some difficulty in explaining how phosphate and lactate enter and leave the muscle. If they do not pass as anions the phosphate might pass in some organic combination and lactate as undissociated lactic acid. The theory also meets difficulty in explaining electrolyte changes in recovery from stimulation for which rather artificial hypotheses have to be advanced. It is necessary, therefore, to use this theory with reservations. Possibly some dynamic theory, such as that suggested by Osterhout (1933), will prove to be better, but at the present time this dynamic theory explains the concentration of potassium inside the cells in terms of a constant diffusion gradient of acid from the inside out and the diffusion of carbonic acid is quantitatively inadequate to fill this rôle.

An electrophoretic theory has been advanced by Keller (1933, 1934, 1935a, b, c) to explain the distribution of electrolytes in tissues. He observed that in a special cataphoresis cell (Fürth, 1925) in the presence of biological materials, the substances, including potassium salts, which are usually found inside cells move toward the anode, while substances found outside cells, including the sodium salts, move toward the cathode. Keller has therefore postulated that the inside of the cells is positive while the tissue spaces are negative. The potential difference then brings about the separation of the potassium and the sodium. Diffusion potentials resulting from the separation would tend to diminish the initial potential. Unfortunately, there is no evidence for a potential difference of this sort which is exactly opposite to the difference supposed to exist between the inside and the outside of muscle cells on the basis of injury potential measurements. Potential measurements quoted by Keller, in which the electrodes are merely placed on the outside of organs for comparison with another electrode in the blood, are difficult to interpret and certainly do not indicate the potential between the inside and the outside of the cells. Moreover, it is hard to see how a membrane potential could distribute electrolytes except within the membrane itself. The theory must therefore be regarded as highly improbable until the necessary potential is proved to exist.

Finally, a theory may be mentioned according to which muscles are impermeable to all electrolytes under normal conditions. Changes in electrolyte content are then explained on the basis of specific functional changes in permeability. This theory has the advantage of great elasticity, but it is so vague as to be little more than a cloak for our ignorance.

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ENDOCRINE CONTROL OF THE MAMMARY GLAND

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During the past few years no phase of experimental endocrinology has been more rapidly developed than that which deals with the hormonic factors involved in the development and function of the mammary glands. The quarter century preceding 1928 saw very little advance in the understanding of lactation phenomena over the purely casuistic analyses of Halban (1905) and the experimental studies of Ancel and Bouin (1911). Since the introduction of a new factor, the anterior pituitary, and the advent of purified ovarian hormone preparations in general use, the experimental laboratories have yielded a steady flow of new facts relating to the endocrine physiology of the mammary glands. A great deal of important progress has been made and we are now in a position to understand many of the factors concerned in the control of lactation, yet many puzzling questions remain. Even now, some of them are being explained; no doubt others will soon be solved. Meanwhile, it may be profitable to examine the position of the general problem. If it is complex, it is only because the problem which originally appeared very simple has been shown, during its evolution under experimental attack, to possess aspects of a totally unforeseen nature.

As indicated in the title, this review limits itself to the hormonic factors concerned in lactation. It would be valuable to consider some of the more purely metabolic and general physiological processes contributing to the secretion of milk, but this falls outside the scope of the present article.

The changes which occur in the female mammary gland may be conveniently divided into six phases, viz., prenatal, birth to puberty, recurring sexual cycles, pregnancy, lactation, and involution. In this review we will consider those changes which normally occur during the sexual cycles, pregnancy, and lactation, and in their experimental reproduction.

In order to furnish a basis for discussion, it may be of interest to review briefly the glandular characteristics of the mammae. The fully developed mammary gland is a compound tubulo-alveolar gland. A variable number of lactiferous or lobar ducts radiate from the nipple and

subdivide in an arborescent fashion into numerous intralobar and interlobular ducts. These in turn give rise to the intralobular ducts along which the true secretory elements, the alveoli, develop. A group of alveoli form a lobule, and a number of lobules constitute a lobe. Figure 1 portrays, in a diagrammatic fashion, the various mammary elements as represented in the rat or mouse gland. Dabelow (1933) has presented an interesting morphological study of the mammary glands and the associated blood vessels of several species.

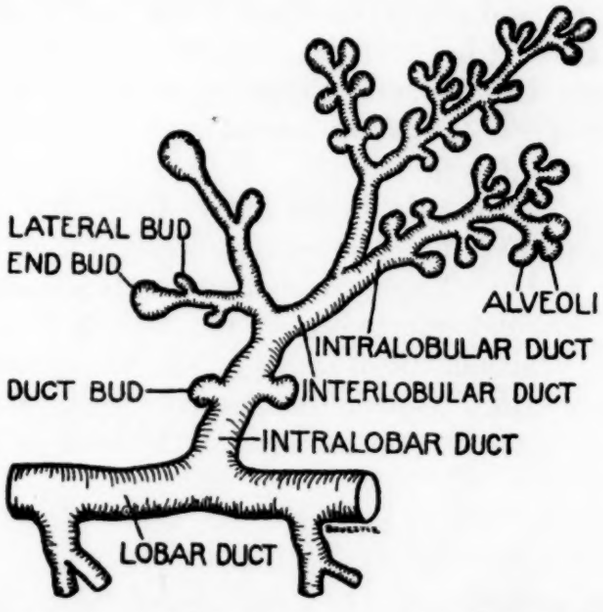

Fig. 1. The left half of the diagram shows the ducts and end buds which characterize the mammary gland before the stimulation of the development of lobules and alveoli has occurred. The right half of the diagram portrays the grouping of alveoli into lobules and is typical of the fully proliferated rat or mouse gland.

NORMAL DEVELOPMENT. *Prepubertal.* During fetal life the development of the mammary glands is much the same in both sexes of most species. The nipples and rudimentary glands are developed from parallel thickenings of the epidermis, the milk lines. Local invagination and sprouting give rise to the individual glands and their ducts, in the numbers and groupings which are characteristic of the various species. Prior to puberty the primary duct system shows a slight further extension which varies in degree between the different species and to a lesser extent between the sexes. However, it is uniformly true that the normal prepubertal development consists only of a more or less progressive

growth of the ducts. There is no evidence of the extensive development of the ducts and the marked lobule-alveolar proliferation which occurs in the later periods. Turner (1932) has reviewed the mammary changes which occur during the early phases, as well as those during involution.

Coincident with the accelerated development of the ovary at the onset of puberty, the female mammary gland undergoes further proliferation. This growth is dependent upon the ovary and is correlated with the succession of sexual cycles which characterize the reproductive lives of most mammals. Exceptions to this cyclic condition are found in a few species, such as the ferret and rabbit, in which the period of oestrus is often greatly prolonged. It has been possible to correlate the changes in the mammary glands with the various types of oestrous cycles and to show that certain changes in the glands depend upon the factors active in the different phases of the cycle.

Acyclic forms. Ancel and Bouin (1911) have shown that a very marked development of the duct system occurs during the prolonged oestrum of the rabbit. This duct proliferation is accompanied by the appearance of terminal enlargements. On the other hand, the ferret (Hammond and Marshall, 1930) shows no significant mammary development during oestrus. As ovulation normally occurs only after coitus in these animals, there is no luteal phase of the cycle in the absence of copulation.

Short cycle forms. The cycle of the rat, which occurs every 4 or 5 days, has only a short follicular or oestrual phase and a luteal phase that is probably insignificant. In a short paper Sutter (1921) reported cyclic changes in the mammary glands which corresponded to the events of the recurring cycles. Turner and Schultze (1931) observed considerable variation in the cyclic condition of the mammary glands. The major portion of the development was attained at the oestrous period and there appeared to be an indication of a limited lobule proliferation with successive cycles.

In the mouse, which has a similar cycle, Cogswell (1928) stated that the mammary glands undergo progressive development through oestrus and metestrus. According to Bradbury (1932b) development and regression, correlated with the cycle, occurs, but the developmental phases tend to be cumulative so that virgin animals a year old show better developed glands than do those only five months old. On the other hand, Gardner and Strong (1935) state that the mammary glands of mice attain their full virginal development at the sixtieth or seventieth day of life. Turner and Gomez (1933a) and Cole (1933) re-

ported that dilatation of the ducts and the appearance of new end-buds occur during oestrus, but that metestrus is accompanied by regressive changes. Gardner (personal communication) has made observations on 43 female mice killed at ages varying from 100 to 350 days and at periods of 15, 30, and 100 days following the cessation of suckling. He has found it impossible to correlate the state of the mammary glands with the stage of the oestrous cycle. From the foregoing studies it would appear that, in the mouse, definite cyclic changes occur in the mammary glands of virgin animals, but that such changes are not detectable after the glands have progressed through pregnancy and lactation.

Long cycle forms. The cycles in this group vary in length from about 15 to 30 days and have a definite follicular phase as well as a luteal phase of considerable duration. Observations on the guinea pig by Loeb and Hesselberg (1917) and Turner and Gomez (1933b), on women by Luchsinger (1927) and Lewis and Geschicter (1934), and on the heifer by Hammond (1927) have shown that in general there is a progressive development of the ducts, and, to a slight extent, the alveoli during the cycle.

Forms exhibiting pseudo-pregnancy. Most of the forms falling in this group have long cycles, but they logically constitute a separate class in view of the unusual character of their metestrus periods. Probably a large number of species exhibit this form of cycle, but only a few have been identified by adequate study. Among these are the dog and two marsupials, the Australian native cat (Dasyurus viverrinus) and the opossum. Ovulation is spontaneous in these animals and in the absence of conception the corpus luteum, unlike that of most forms, is maintained for a period of time equivalent to that of pregnancy. It has been shown for the dog by Marshall and Halnan (1917) and Turner and Gomez (1934b), for Dasyurus by O'Donoghue (1912), and for the opossum by Hartman (1923) that the mammary glands, during the prolonged metestrum, proliferate in a fashion that is entirely equivalent to the development during pregnancy. Indeed, in the case of the dog, at least, lactation to a degree which allows for the suckling of a litter may occur at the close of pseudo-pregnancy.

Sterile copulation in many animals is followed by a prolonged luteal phase during which the mammary glands show the same type of proliferation as in the first half of pregnancy. In most species, e.g., the rat and rabbit, this period of pseudo-pregnancy has a duration which is roughly equivalent to one-half the length of pregnancy. In the ferret

(Hammond and Marshall, 1930) pseudo-pregnancy is equivalent to pregnancy in duration and the mammary changes run parallel courses in both conditions. For a further discussion of the subject the reader may be referred to the papers by Turner and Gomez (1933a, 1934b). The term "complete pseudo-pregnancy," has been suggested for the condition in the dog, ferret, etc., while the shorter period in such animals as the rabbit and rat, has been called "incomplete pseudo-pregnancy."

In summarizing, the character of mammary proliferation during the oestrous cycle varies considerably from one species to another. It can be rather well correlated with the type of cycle and shown to be parallel to the degree of follicular and luteal activity. In animals whose cycle is characterized by a persistent corpus luteum, the mammary gland undergoes extensive development, i.e., to the compound tubulo-alveolar stage. In instances where the luteal phase is either absent or of short duration the glandular proliferation extends the duct system with little or no development of the lobule-alveolar system. In other words, the gland is not carried beyond the compound tubular condition.

Pregnancy. Emphasis has been placed upon the advanced type of mammary development which is associated with a prolonged luteal phase of the reproductive cycle. During pregnancy the corpora lutea of all animals reach the height of their activity and it has been uniformly observed that the mammary glands show a progressive duct and lobule development. The glands thicken and as pregnancy reaches its later stages the alveoli show evidence of secretory activity. With the events of parturition, lactation is initiated and continues for a period in keeping with the requirements of the offspring. The concomitant development of the corpora lutea and the mammary glands early led to the belief that proliferation of the latter during pregnancy depends entirely upon the hormone of the corpus luteum. As shall be shown presently, later studies have complicated this relatively simple explanation and have shown that certain other hormones are involved.

Turner (1932) has reviewed the earlier reports on mammary development in pregnant animals. More recent studies include those of Bradbury (1932a, b) and Turner and Gomez (1933a) on the mouse, Turner and Gomez (1933b) and Nelson (1934a) on the guinea pig, Deanesley (1934) on the hedgehog, Deanesley and Parkes (1933) on the grey squirrel, Turner and DeMoss (1934) on the cat, and Turner and Gomez (1934b) on the dog.

Hammond (1927) in considering the proliferation of the mammary glands during pregnancy in the cow portrayed two phases of development. During the first five or six months the entire lobule-alveolar system is formed through progressive proliferation of the ducts and end-buds. From the sixth month until parturition the alveoli undergo secretory activity resulting in the accumulation of secretory products and a swelling of the entire glandular system. This enlargement or hypertrophy has led many investigators to believe that actual growth or hyperplasia of the gland occurs during the later stages of pregnancy. Thus in animals which have a pseudo-pregnancy equivalent in length to about one-half the gestation period, the mammary changes associated with pregnancy and pseudo-pregnancy run parallel courses. In addition, the mammary glands of the pregnant animal go through a superimposed secretory phase which is much less marked during pseudo-pregnancy. It should be noted that Weichert, Boyd, and Cohen (1934) do not consider the mammary development during the first half of pregnancy to be equivalent to the pseudo-pregnant development in the rat. They tend to the view that the latter is an abbreviated pregnancy insofar as the activity of the mammary glands is concerned, since they observed a typical secretory phase at the close of pseudo-pregnancy. Roberts (1921), Parkes (1929b), Bradbury (1932b) and Turner and Frank (1932) also have emphasized the division of mammary development during pregnancy into proliferative and secretory stages.

The male mammary gland. Development of the male mammary gland after birth is usually limited to a slight extension of the duct system. The rat (Turner and Schultze, 1931), man (Cooper, 1845; Andrews and Kampmeier, 1927), and probably the goat are exceptions, often showing a progressive and rather extensive proliferation of the ducts. The guinea pig and monkey have a slight development, while the mouse, rabbit, dog, cat, and other investigated species show little or no extension of the duct system. For this reason the male animal offers an excellent subject for examining the effects of various hormone preparations upon the mammary gland. It has been definitely shown that the male mammary gland has the capacity for response to growth-promoting substances and that the absence of development in the normal male may be attributed to a deficiency of the ovarian hormones associated with mammary growth (Nelson and Pfiffner, 1931; Nelson and Smelser, 1933; Turner and DeMoss, 1934). It is interesting to note that demonstrable amounts of oestrogenic hormone are commonly present in the urine of men (Zondek and Euler, 1934), and have been detected in the excreta of male rats (Turner, 1932).

EXPERIMENTAL DEVELOPMENT OF THE MAMMARY GLAND. A résumé of the vast array of earlier papers dealing with the observations made on the changes induced in the mammary glands by various preparations of whole ovaries, corpora lutea, and placentae will not be attempted in the present article. The more important references may be found in the summaries by Parkes (1929a) and Turner (1932). Most of these observations on the experimental development of the mammary glands were purely incidental to the investigators' major object, viz., a study of the uterus, and it is now apparent that such active material as was present in most of the extracts almost certainly was the oestrogenic hormone. Subsequent experiments carried out with pure oestrogenic hormone[1] leave little doubt that it is the substance responsible for the mammary effects observed in the earlier studies.

Effect of oestrone. It has been clearly shown that species differences exist in the response of the mammary glands to oestrone injections. In males and spayed females of all species it induces growth of the ducts, i.e., a proliferation similar to that observed in the oestrous phase of the cycle. There is also uniformity in the reports of a stimulation of nipple growth. However, distinct differences occur between the various animals in the response of the lobule-alveolar system to oestrone. In the mouse there is no growth of the lobules (Bradbury, 1932a, b; Turner and Gomez, 1934). In the rat Turner and Schultze (1931) found only limited proliferation, while Selye, Collip and Thomson (personal communication) and Nelson (1935c) have observed very considerable development of the lobules. In the cat (Turner and DeMoss, 1934) and the dog (Turner and Gomez, 1934) oestrone will induce only an extension of the duct system. Indeed, in the dog as much as 4000 rat units given over a period of 30 days induced only a slight growth of the ducts. In contrast are the experiments of Kunde, D'Amour, Carlson, and Gustavson (1930). In their study of the effect of oestrone on basal metabolism, 2 spayed and 2 normal dogs were treated with 100 to 200 rat units daily for 39 days. Nipple growth was observed in all animals, but no examinations of mammary tissue were made. One normal animal began lactating five days after the initiation of treatment and successfully suckled a second dog's litter. It is to be noted, however, that the protocol shows that this animal, the only one to lactate, had been in oestrus about 63 days earlier.

[1] In discussing the effects of the oestrogenic hormone the term "oestrone" will be employed. This term, attractive for its brevity and appropriateness, was suggested by Adam et al. (1933) for ketohydroxyoestrin, which has been known under the various names of theelin, oestrin, menformon, folliculin, etc.

As noted in an earlier section of this article, the mammary glands of the unmated dog undergo complete proliferation in the nine week period corresponding to pregnancy. Consequently, it is unlikely that the injections of oestrone were responsible for the observed lactation. Houssay (1935b) has reported that oestrone (1000 to 10,000 international units daily for 49 to 90 days) induced marked development of the glands and nipples of 3 male dogs. No details were given regarding the type of proliferation present in the glands.

The rabbit, according to Parkes (1930) and Turner and Frank (1932) shows marked extension of the duct system after administration of oestrone. There was very little proliferation of lobules in animals receiving as much as 2400 rat units distributed over a period of 120 days. However, Frazier and Mu (1935) have recently reported experiments which would indicate that proliferation of the lobule-alveolar system may occur. Lyons (personal communication), too, has evidence which indicates that oestrone is the only ovarian hormone needed for alveolar development in the rabbit.

In the guinea pig it has been clearly shown that oestrone will induce complete development of the lobule-alveolar system as well as an extension of the ducts. Haterius (1928), employing an aqueous extract prepared from placentae, produced a marked development of the ducts and lobules. Similar results were reported by Laqueur, Borchardt, Dingemanse, and de Jongh (1928) following the injection of 30 to 300 mouse units of menformon in oil over a period of 26 days. Nelson and Smelser (1933), injecting 375 rat units of oestrone in oil, distributed over a period of 11 weeks in male guinea pigs, were able to demonstrate that oestrone alone produces complete development of the lobule-alveolar complex. These experiments have been considerably extended by Nelson (1936). It has been shown that as little as 5 rat units per day of a preparation of oestrone in oil over a period of 10 weeks would induce in males and spayed females a development of the mammary gland comparable to that observed in females near the close of pregnancy. On the other hand, as much as 50 rat units daily of an aqueous preparation of oestrone produced only a partial proliferation of the lobules. Turner and Gomez (1934a) also have observed marked proliferation of the mammary glands in males injected with oestrone in oil. In the goat, De Fremery (1934) has reported that oestrone alone will prepare the glands for lactation. However, Turner and Gomez (personal communication) have observed lactation in young female goats which have received 100 r.u. of oestrone daily for three weeks. Section of the glands revealed only proliferation of the ducts.

Few reports have appeared concerning the effect of oestrone on the mammary glands of monkeys or of man. Allen (1927) and Allen, Gardner, and Diddle (1935) noted an extensive development of the duct system in spayed and normal female monkeys following injections of aqueous oestrone. There was some evidence of alveolar proliferation, but this was not judged to be extensive enough to permit the conclusion that oestrone alone would develop the lobule-alveolar system. However, the periods of treatment were relatively short and, as noted above, were conducted with aqueous oestrone. Turner and Allen (1933) reported that the injection of 14,000 rat units of a preparation of oestrone in oil over a 65 day period in a male monkey induced extensive lobule formation. In one male and two immature female monkeys following the daily administration of 100 to 200 rat units of oestrone in oil for 4 to 7 months, the reviewer has observed distinct evidence of lobule as well as duct proliferation. However, even in these instances the glands did not attain the degree of development seen in pregnancy.

Obviously, great difficulties attend the study of experimental development of the mammary glands in man. Such reports as have appeared are not conclusive since actual study of the mammary glands was not made. It is reported that distinct enlargement of the breasts, accompanied by a tingling sensation and a feeling of fullness, occurs in spayed women following treatment with oestrone. The observations of Werner and Collier (1933) and Werner (1935) are examples of these reports. However, there are certain observations of an indirect nature which are suggestive. The reports of gynecomastia in men might indicate that oestrone alone can induce extensive mammary development. Furthermore, it has been repeatedly reported that pregnancy will continue in women after oöphorectomy and that both delivery and lactation are entirely normal. Since oöphorectomy removes the corpus luteum, and since it has been shown that the guinea pig, which does not require the corpus luteum hormone for complete mammary development, will also maintain pregnancy in the absence of the ovaries, it is possible that the corpus luteum hormone is not required for the development of the human mammary gland. However, in all these instances there is no conclusive evidence that progesterone, derived from an extra-ovarian source, was not present.

In summary, there are distinct species differences in the response of the mammary glands to oestrone. At one extreme are the dog and mouse, which show only duct stimulation, and at the other the guinea pig, which exhibits proliferation of all mammary elements. It should be noted here that most of the experiments have been conducted with

oestrone (ketohydroxyoestrin) in varying degrees of purity. Turner, Frank, Gardner, Schultze, and Gomez (1932) found no significant differences between oestrone and oestriol (trihydroxyoestrin, theelol) in their effects on the mammary glands of mice, rats, and rabbits. Nelson (1936) has made similar observations on the guinea pig. As yet few reports have appeared concerning the effect of the many compounds, physiologically and chemically related to oestrone, on the mammary glands. Lacassagne (1932) observed the occurrence of mammary carcinoma in male mice following the administration of folliculin benzoate. Details concerning the exact nature of the changes in the duct and lobule-alveolar systems are lacking, but it seems probable that considerable development of the lobules occurred. Burrows (1935) has compared the effects of several oestrogenic substances on the mammary glands of male mice. Oestrone produced only a wide extension of the duct system with little proliferation of alveoli. On the other hand, equilin caused little or no extension or dilatation of the ducts, but induced a marked proliferation of the lobule-alveolar system. More recently Burrows (personal communication) has found that certain of the dibenzanthracene compounds and ketomethoxyoestrin produce effects similar to oestrone, while oestradiol (dihydroxyoestrin), like equilin, induces alveolar proliferation. Bradbury (personal communication) too, has observed considerable extension of the duct system in male rats and mice injected with a dibenzanthracene derivative. Gardner, Smith, and Strong (1935) have found that folliculin benzoate produces the same type of alveolar hyperplasia as was observed by Burrows. Furthermore, they confirmed the findings of Lacassagne on the occurrence of mammary carcinoma as an aftermath of the alveolar stimulation. The relation of oestrogenic substances to mammary cancer is an absorbing question, particularly in view of the above findings, which seem to indicate that the prolonged administration of certain oestrogenic compounds will lead progressively to proliferation of the normal glandular elements, adenomata, and finally carcinomatous growths which are highly malignant. Although various patterns of mammary growth have been produced by different oestrogenic substances, the quantitative factor has not been ruled out at the present time. Gardner, Smith, and Strong (1935) have emphasized the occurrence of varying degrees of mammary proliferation, from simple duct growth to the proliferation of markedly atypical tissue, in the different parts of a single gland in mice receiving folliculin benzoate. Such a condition, quite correctly, is considered abnormal and is unlike the

uniform type of development normally present in glands which are proliferating under the stimulus of the animal's own ovarian hormones. A further consideration of the relation between oestrogenic and carcinogenic substances is impossible here, but the reader may be referred to the recent review by Loeb (1935).

Effect of progesterone. In referring to the hormone of the corpus luteum the recently adopted term "progesterone" (Allen, Butenandt, Corner, and Slotta, 1935) will be employed. This term is more appropriate than the older ones (progestin, luteosterone, corporin, lutin) and is in keeping with the usage of the term "oestrone."

Many of the earlier studies referred to the effect of corpus luteum extracts upon the mammary glands. However, it now seems probable that such active material as was present in these extracts was an oestrogenic substance. In those studies in which the guinea pig was the experimental animal definite mammary growth was observed. These results were considered to support the contention (Ancel and Bouin, 1911; Hammond, 1917) that the corpus luteum is responsible for the development of the mammary glands during pregnancy. However, it is now known that oestrone alone induces this type of proliferation in the guinea pig. The reader is referred to the articles by Nelson and Pfiffner (1931) and Turner (1932) for a summary of these earlier studies.

Some indirect evidence for the participation of the corpus luteum in the development of the mammary gland was adduced by Parkes (1929b). He injected pseudo-pregnant rabbits with an alkaline extract of the anterior pituitary and noted that the existence of the corpora lutea was prolonged. Coinciding with the extended luteal phase there were changes in the mammary glands which duplicated those seen in the latter half of pregnancy and after parturition, viz., thickening of the glands, initiation of the secretory phase, and actual lactation. These effects were attributed to the continuation of luteal activity through the gonadotropic property of the pituitary extract. Similar results were observed on the rat by Evans and Simpson (1929). As will be shown later, an additional and unsuspected factor, the direct action of the pituitary extract on the mammary glands, was important in the production of these changes.

Allen and Corner (1929) were able to show that lipoid extracts of sows' corpora lutea would induce changes in the uterus of the rabbit characteristic of early pregnancy, and furthermore, would maintain the life and development of the embryos in rabbits spayed early in pregnancy. These effects were never observed in animals receiving

oestrone and established on an experimental basis the existence of a hormone produced by the corpus luteum. Inasmuch as the pregnant rabbits which had received progesterone showed entirely normal development of the mammary glands and lactated after parturition, Corner reasoned that if these functional changes were actually directly due to progesterone it should be possible to duplicate them in spayed non-pregnant animals. When this idea was put to test (Corner, 1930) it was observed that no significant alteration of the mammary glands occurred. Furthermore, he observed that the prolongation of luteal activity through the induction of ovulation and corpus luteum formation at 10 day intervals failed to produce mammary changes beyond those present in ordinary pseudopregnancy. Nelson (1932a) confirmed and extended these observations by experiments in which new groups of corpora lutea were formed at weekly intervals, and, at the same time, the uterus was traumatized. This led to the formation of decidual tissue which it was believed might be the missing factor in Corner's experiments. However, no further development of the mammary glands was observed. Asdell and Hammond (1933) observed that hysterectomy in the rabbit would prolong the life of the corpora lutea, but that the mammary glands showed no development beyond the usual condition of pseudo-pregnancy.

Nelson and Pfiffner (1930, 1931) observed that corpus luteum extracts would induce lobule proliferation in male or spayed female rabbits, rats, and guinea pigs. It was believed that these extracts were relatively free of oestrogenic hormone as vaginal cornification was never observed in spayed rats following their injection. However, in view of additional data it now seems likely that some quantity of oestrone was present, but masked in the rat tests by the inhibitory action of progesterone. Recent experiments conducted with the relatively crude type of extract and extracts from which the oestrone had been removed by additional procedures have shown that the purified material will not induce significant mammary changes in the absence of oestrone.

Turner and Schultze (1931) and Turner and Frank (1932) observed that the administration of progesterone in the rat and rabbit had little effect on the mammary glands. However, these workers were able to induce definite formation of lobules by the simultaneous injection of progesterone and oestrone in animals that had first been subjected to preliminary treatment with oestrone. That the use of this hormone sequence, i.e., oestrone followed by progesterone and oestrone, would produce development of the lobule-alveolar system has since been

shown in the rabbit by Riddle, Bates, and Dykshorn (1933) and Asdell and Seidenstein (1935), in the mouse by Turner and Gomez (1934a), in the cat by Turner and DeMoss (1934), and in the rat by Freud an De Jongh (1935). It should be emphasized that in the above hormone sequence the preliminary treatment with oestrone serves to induce growth of the ducts and perhaps to sensitize the end-buds to stimulation by progesterone. The progesterone-oestrone phase results in an extensive multiplication of alveoli and produces a mammary gland typical of mid-pregnancy or the end of pseudo-pregnancy in the rat or rabbit. It is to be noted that the secretory phase which occurs in the latter part of pregnancy has not been reported following the above procedures.

Attention has been called to the capacity of oestrone to stimulate development of the lobule-alveolar system in the guinea pig. In the reviewer's experience corpus luteum extracts are ineffective in stimulating development beyond that obtained with oestrone alone. This is particularly true of oil preparations of oestrone. In animals treated with moderate amounts of aqueous oestrone the subsequent administration of progesterone does induce further development (Nelson and Pfiffner, 1931; Nelson, 1936). Turner and Gomez (1934a) noted a few cases in which the simultaneous injection of progesterone and oestrone induced distention of the alveoli, i.e., the alveolar change which occurs normally in the latter part of pregnancy.

No reports have appeared concerning the effect of progesterone on the monkey's mammary gland, and only one on human subjects, that of Werner (1935). Two castrate women treated with progesterone and oestrone were said to have shown greater breast enlargement than others receiving oestrone only. Since no material for histological study was available it is difficult to evaluate this report.

In summarizing the relation of progesterone to mammary proliferation it may be said that the weight of evidence at this time indicates that in combination with oestrone it induces development of the lobule-alveolar system of most animals to the condition seen in mid-pregnancy. The guinea pig gland is an exception as progesterone produces little, if any, change beyond that induced with oestrone only. However, in view of the close chemical relation between progesterone and the various oestrogenic compounds, and of Burrows' demonstration that some oestrogenic substances do induce development of the lobules in the mouse, it is impossible at this time to properly evaluate the exact status of the ovarian hormones in their rôle as stimulators of mammary development.

The effect of ovarian grafts. No review of lactation would be complete without a reference to the effect of ovarian grafts upon the mammary gland. Guinea pigs have been a favorite subject for this type of study, although the rat (Engle, 1929), rabbit (Kallas and Lipschutz, 1929), mouse (Gardner, 1935), and heifer (Turner, 1931) have been employed. The early literature on this subject has been reviewed by Moore (1921) and Smelser (1933). It has been observed that successful ovarian grafts would stimulate the atrophic mammary glands of castrated females and lead to a marked development of the male gland. Indeed, the male guinea pig shows a much greater degree of mammary proliferation than the female (Lipschutz, 1927). This has been attributed to an inherently greater gonad-stimulating capacity of the male hypophysis which initiates a greater and more constant production of oestrone by the grafts in males (Smelser, 1933; Nelson, 1935a). It is significant that ovarian grafts in male animals almost invariably contain only follicles, corpora lutea occurring only rarely. Lactation may be induced in such animals by injections of anterior hypophyseal extracts (Nelson and Smelser, 1933) and occurs without treatment if the graft ceases to function, either spontaneously or through its surgical removal. This is probably due to the removal of the lactation-inhibiting influence of oestrone (Nelson, 1932b).

THE RELATION OF THE ANTERIOR PITUITARY TO LACTATION. Up to this point direct references to lactation have, in the main, been avoided, although many of the quoted studies dwelt to a greater or lesser degree upon that phenomenon, in order to fully establish the purely developmental phases of mammary activity. We now pass to a consideration of what may be termed the differential phase, lactation.

Most of the attempts to induce lactation prior to 1928 had met with failure. The earlier workers considered lactation to be a process which occurred as the glands reached the height of their development and when the influences which had promoted growth were removed. Thus, the placenta, fetus, and corpus luteum have at various times been looked upon as the controlling factor whose activity and removal or loss of function, in turn, promoted mammary growth and permitted lactation. The initiation of milk secretion was, therefore, believed to be in the nature of a purely catabolic process, and without an active stimulus. However, repeated attempts to induce lactation by experiments designed with this theory in mind met with little success. Favorable results were obtained in the guinea pig following the discontinuation of oestrone injections (Steinach, Dohrn, Schoeller, Hohlweg,

and Faure, 1928; Laqueur, Borchardt, Dingemanse, and de Jongh, 1928). Although Fellner (1931) believed that similar experiments on the rabbit supported the above theory most of the evidence indicates that the rabbit gland undergoes duct proliferation only with such treatment. It will be recalled that the guinea pig gland responds completely as a result of treatment with oestrone.

Meanwhile an important contribution from Bouin's laboratory at Strasbourg introduced an entirely new factor into the lactation problem. Stricker and Grueter (1928) announced that they had been able to induce copious lactation in rabbits by the use of extracts of the anterior pituitary. Later they reported the stimulation of lactation in bitches, sows, and cows (Grueter and Stricker, 1929). This important discovery showed that the initiation of milk secretion depends upon an active stimulus rather than the cessation of growth processes induced by the ovarian hormones. It will be shown that this latter event is important in the initiation of lactation, but not in the same manner as had been supposed.

Corner (1930), unaware of Stricker and Greuter's work, also discovered that lactation could be initiated in the rabbit with anterior lobe extracts. Following his failure to obtain significant mammary development with progesterone he reasoned that Parkes' (1929b) success might have been due to a direct action of the pituitary extract on the mammary gland rather than to stimulation of the corpora lutea. On putting this idea to test he found that spayed virgin rabbits injected with an alkaline extract of sheep pituitaries lactated copiously. In certain details, such as the relative necessity for luteal stimulation of the mammary glands preliminary to pituitary action, the results of Corner and of Stricker and Greuter differed, but on the main issue, that of the lactogenic properties of anterior lobe extracts, there was no disagreement. Subsequent work has firmly established this reaction in many animals.

Nelson and Pfiffner (1930), using fresh pituitary implants and extracts similar to those employed by Corner, were able to initiate lactation in normal and spayed female guinea pigs and in males or immature females which had been treated previously with oestrone and progestrone. It was also shown that the mammary glands of males and immature females which had not received preliminary treatment with the ovarian hormones failed to respond to the pituitary extracts. Thus, some degree of glandular preparation by ovarian hormones was required before the lactation-inducing stimulus of the hypophysis was effective.

Subsequent studies have fully confirmed this relationship. The experiments on guinea pigs were continued and extended to rabbits and rats with essentially the same results (Nelson and Pfiffner, 1931).

Asdell (1931) confirmed Corner's work with spayed rabbits and also succeeded in bringing into lactation a young goat which had never been in heat. Later Asdell (1932) showed that administration of the pituitary extract to goats in advanced lactation would prevent for a time at least the normal decline of milk yield. Turner and Gardner (1931) advanced further evidence that the injection of anterior hypophyseal extracts would stimulate lactation in the mature female rabbit. Bradbury (1932b) observed secretory changes in the mammary gland of the mouse after the administration of a pituitary extract.

Before proceeding to the very considerable body of papers which have appeared since the pioneer studies described above, it may be of value to digress long enough to note the nature of the mammary response to pituitary extracts containing the lactogenic hormone. Presumably some alveolar development was present in the glands of all the animals that lactated in the above experiments, although it is now apparent that under certain conditions the ducts may produce milk or a milk-like secretion (Weichert, Boyd, and Cohen, 1934; Gardner, Gomez, and Turner, 1935). Thus actual production of milk in from 3 to 6 days was a regular and predictable occurrence in properly prepared animals. Moreover, it was believed that the pituitary extracts also induced growth of the glands by a direct action. This direct stimulation of mammary growth is, of course, quite separate from the proliferation induced by any ovarian hormones which might have been secreted as a result of the gonadotropic activity of the extracts. It is quite true that the mammary glands show a definite enlargement and thickening in the spayed as well as the intact animal, following a course of injections with the extracts. However, it would seem that this growth is more apparent than real, as a critical study of glands removed before and after induced lactation reveals no proliferation of new tissue, but only an engorgement of the pre-existing mammary elements with milk. Attention was called to this condition by Turner and Gardner (1931) and it has been emphasized further by Turner (1932), Gardner and Turner (1933), and Nelson (1934b). It should be noted that Lyons and Catchpole (1933b) support the earlier belief that actual mammary proliferation is induced by the lactogenic hormone.

The stimulation of lactation by anterior hypophyseal extracts has been reported in a number of other animals. These include the goat

(Evans, 1933), sow (Gardner and Turner, 1933), dog (Lyons, Chaikoff and Reichert, 1933), heifer (Catchpole, Lyons, and Regan, 1933), cat (Turner and De Moss, 1934), ground squirrel (Nelson, 1934b), monkey (Riddle, Bates, and Dykshorn, 1933; Allen, Gardner, and Diddle, 1935), and the ferret (McPhail, 1935a).

THE INITIATION OF LACTATION. For many years it was supposed that lactation results as a natural consequence of the cessation of mammary development, i.e., when the stimulus for growth is removed the alveolar cells begin to secrete. Considerable experimental evidence has been offered for this general theory. The experiments of Hammond (1917) and Drummond-Robinson and Asdell (1926) led them to believe that milk secretion occurred when the corpora lutea were removed or when they ceased functioning, provided that the mammary glands had attained a certain degree of development. A group of workers, using the guinea pig, reported that the cessation of oestrone injections was followed by lactation and they were led to consider oestrone as the hormone of lactation. The paper by de Jongh and Laqueur (1930) is an example of these studies on the guinea pig. Fellner (1931), on the basis of experiments on rabbits and guinea pigs, offered further support for this theory. It is noteworthy that although these and other similar papers were published after Stricker and Grueter's original accounts of a pituitary lactogenic hormone had appeared, little or no significance was attached to a pituitary participation in lactation. Thus it was believed that lactation results, as a passive process, whenever the growth-promoting factors are removed.

Corner (1930), aware of these findings and convinced of the importance of the pituitary as an active promoter of lactation, suggested that some factor restrains lactation during pregnancy. Nelson and Pfiffner (1931), realizing the necessity of harmonizing the pertinent factual knowledge, suggested that the placenta might produce the substance which inhibits lactation. Further studies have indicated strongly that such may be the case.

In a series of papers (Nelson, 1932b, 1933, 1934a, 1935a; Nelson and Smelser, 1933) a hypothesis was developed which took into account the growth-promoting rôle of the ovarian hormones and the lactogenic property of the pituitary in the sequence of events which mark the development and function of the mammary glands. In short, this idea is that during pregnancy the ovarian hormones induce the proliferative changes that occur in the mammary glands, but that during this time, particularly during the latter phases of pregnancy when the glands

are fully prepared for lactation, they inhibit the actual secretion of milk. This restraining influence was visualized as operating through two channels, viz., by suppressing the secretion or release of the lactogenic hormone from the pituitary and by a direct inhibitory effect on the mammary glands. Thus the high ovarian hormone content during pregnancy would be conducive to glandular development, but inhibitory to lactation. With the decline in the level of the ovarian hormones at parturition, the inhibitory influences would be removed, the lactogenic hormone would be secreted, and lactation would occur. The inhibitory factor is believed to be principally oestrone, but some evidence (Nelson, 1934a; Donahue, 1934) points to a possible participation of the corpus luteum as well.

Space does not permit an account of all the evidence offered in support of this hypothesis, but the following summary of the experiments on pregnant guinea pigs may be of value: 1. The injection of purified lactogenic hormone into pregnant animals failed to induce lactation or to affect the pregnancy. 2. Crude preparations did induce lactation, but only after pregnancy was terminated by abortion. 3. Extirpation of the ovaries was not invariably followed by abortion. Lactation occurred only after fetal expulsion whether abortive or at term. 4. Surgical removal of the entire uterus with the fetuses did not lead to lactation unless lactogenic hormone was injected. 5. Surgical removal of the uterus, fetuses, and ovaries did induce lactation. 6. Removal of the ovaries and the fertile uterine horn only, in cases of unilateral pregnancy, resulted in lactation. 7. Lactation did not occur when the ovaries and embryos were removed, leaving the placentae intact, during the period of placental retention, but did occur following expulsion of the placentae. 8. Lactation was inhibited in normal parturient females by the injection of oestrone. If lactogenic hormone was administered simultaneously with oestrone, lactation occurred. However, the oestrone dosage could be increased to the point where large amounts of lactogenic hormone failed to stimulate lactation.

These experiments were further supported by various manipulations of non-pregnant female and male animals. Meanwhile other studies were indicating a similar relationship. Smith and Smith (1933) observed that the retention of placental remnants, in parturient rabbits, inhibited lactation and that the injection of oestrone would prevent lactation in rabbits at the close of pregnancy or pseudo-pregnancy. Selye, Collip, and Thomson (1933a) injected female rats with an extract of pregnancy urine and observed marked proliferation of the mammary

glands as an accompaniment of ovarian stimulation. When the hypertrophied ovaries were removed lactation occurred, but when hypophysectomy was also performed no milk was secreted. These experiments were interpreted as supporting the hypothesis outlined above (Collip, Selye, and Thomson, 1933b). Enzmann and Pincus (1933) injected lactating mice with an extract of pregnancy urine and noted a decline of milk secretion as measured by the growth curves of the sucklings. That this decline probably was due to the oestrone produced by the stimulated ovaries is indicated by the report of Robson (1935) who observed a similar failure of lactation in mice injected with oestrone. However, de Jongh (1933) observed that although oestrone inhibited lactation in rats suckling litters, the same result occurred when gonadotropic extracts were injected in spayed as well as in normal lactating rats.

It is interesting to recall the studies of Gaines and Davidson (1926) on milk production in the cow during pregnancy. They noted the usual decline in milk production with advancing pregnancy and the rapid rise at parturition. Although the then existing state of knowledge of the hormones did not permit them to specifically relate any definite factor to the events they had observed, it is significant that they explained the decline in milk flow on the basis of an unknown inhibiting hormone and its rise on some other hormone.

Obviously, experiments having an important bearing on the general theory that removal of the inhibitory influences of oestrone permits the secretion and action of the lactogenic hormone would include removal of the hypophysis in suitable animals. We now pass to a consideration of such studies.

THE EFFECT OF HYPOPHYSECTOMY UPON LACTATION. Allen and Wiles (1932) reported that hypophysectomy in cats near term was followed by normal deliveries, but that milk secretion did not occur. Similar observations on the failure of lactation in rats hypophysectomized during pregnancy were made by Pencharz and Long (1933). Selye, Collip, and Thomson (1933b, c) reported that rats and mice hypophysectomized about the middle of pregnancy would lactate for a few hours after delivery. In similar experiments, Bergman (1934) records the complete failure of lactation. According to Pencharz and Lyons (1934) a transient lactation may occur after delivery in guinea pigs hypophysectomized during pregnancy. Nelson (1935b) found no milk secretion in guinea pigs that had been operated upon during the last week of pregnancy, providing that the hypophysis had been com-

pletely removed. In male and female guinea pigs, in which the mammary glands had been proliferated by ovarian grafts or injections of oestrone, termination of the injections or removal of the grafts was not followed by lactation if the pituitary was removed. McPhail (1935a, b) observed that hypophysectomy during late pregnancy in the ferret and cat was sometimes followed by the birth of living young, but that the mammary glands failed to secrete milk. Houssay (1935a) reports that hypophysectomy interrupts pregnancy in the dog and prevents lactation.

Observations on the effect of hypophysectomy upon established lactation further strengthen the belief in the positive character of the lactation-inducing stimulus exerted by the pituitary. Rapid cessation of milk secretion has been noted in lactating rats and mice (Collip, Selye, and Thomson, 1933a; Selye, Collip, and Thomson, 1933c), in ferrets and cats (McPhail, 1935a, b), in guinea pigs (Nelson, 1935b), in dogs (Houssay, 1935a), and in goats (Hill, Turner, Uren, and Gomez, 1935).

The evidence just outlined is in harmony with the concept that during pregnancy lactation is held in check by some inhibiting mechanism. Elimination of this inhibiting factor, believed to be oestrone or an allied substance, permits lactation providing the pituitary is intact. Thus there would appear to be an interplay between the ovarian hormones which, while furnishing a growth stimulus for the mammary gland, prevent lactation, and the lactogenic hormone, which is concerned with the initiation and maintenance of lactation.

THE UTERUS AS A FACTOR IN THE CONTROL OF LACTATION. However, intriguing as the foregoing explanation may be, there are other factors which introduce complications. It has been known for many years that removal of the fetuses, either by abortion or operation after a certain stage of pregnancy, is followed by lactation (Halban, 1905; Drummond-Robinson and Asdell, 1926). However, it has been noted that if the placentae are not removed lactation does not occur (Halban, 1905; Smith and Smith, 1933; Nelson, 1934a). Selye, Collip, and Thomson (1934; 1935) in addition to confirming these findings on the rat, have carried their examination of the problem a step further. After removing the fetuses and placentae by Caesarian section they immediately filled the uterus with paraffin. Examination of the mammary glands two days later revealed a complete lack of milk secretion. On this basis they suggest that a purely mechanical explanation may account for the suppression of lactation in cases of placental retention.

Loeb (1927) observed that hysterectomy in the non-pregnant guinea pig led to persistence of the corpora lutea and to marked development of the mammary glands. DeJongh (1934) stated that hysterectomy increased the incidence of lactation in virgin rats injected with oestrone. On the other hand, Bradbury (1932a) was unable to stimulate development of the mammary glands in hysterectomized mice. Selye, Collip, and Thomson (1934) on the basis of their observations that a transient lactation occurs at parturition in rats and mice hypophysectomized during pregnancy, believe that the "pregnant uterus or its contents can play the part of the hypophysis in supplying functional stimuli to the mammary glands." These experiments might as easily have been interpreted on the basis of a release of the mammary glands from the inhibitory influence of oestrone. A logical explanation for the transient lactation is found in the recent report of Bates, Riddle, and Lahr (1935) that the hypophyses of fetal calves contain relatively large amounts of lactogenic hormone. It is conceivable that in the absence of the mother's hypophysis the fetal gland would furnish the hormone necessary to initiate lactation. However, Selye (personal communication) has affirmed the idea that the uterus may furnish a positive stimulus for lactation. He has hypophysectomized rats at mid-pregnancy and at the same time removed the fetuses. In these animals a transient milk secretion appeared at about the time parturition would have occurred normally. Furthermore, it is reasonable to question the possibility of the transmission of the hormone from the fetus to the mother.

Nevertheless, emphasis should be placed upon the need for further exploration of the possible relation of the uterus to mammary development and function.

THE EFFECT OF HYPOPHYSECTOMY UPON THE DEVELOPMENT OF THE MAMMARY GLAND. Earlier in this review (p. 503) attention was called to the type of response evoked in the mammary gland by anterior pituitary extracts. Although it was originally supposed that actual proliferation of the glands occurred *pari passu* with lactation, recent opinion has shifted to the belief that the lactogenic hormone is concerned only with actual secretion of milk and does not directly stimulate glandular proliferation. Obviously, important evidence on this point involves the use of hypophysectomized animals in order to determine the character of the mammary response to the ovarian hormones in the absence of any participation of the pituitary.

The findings on the continuation of mammary development in ani-

mals hypophysectomized during pregnancy (p. 506) would appear to be pertinent evidence, as is the report of Jeffers (1935a, b) on the cytological changes in the glands of normal and hypophysectomized pregnant rats. However, the mammary glands of these animals had attained most of their development before the pituitaries were removed. Moreover, the possible participation of the fetal hypophyses cannot be disregarded.

The results of the administration of ovarian hormones to hypophysectomized animals (males or non-pregnant females) have been conflicting. Ruinen (1932) reported that oestrone would induce the same marked development of the mammary glands in hypophysectomized and in normal male rats. Asdell and Seidenstein (1935) on the rabbit, and Freud and de Jongh (1935) on the rat, found progesterone and oestrone as effective in the hypophysectomized as in the intact animal. This finding has been affirmed by Laqueur (personal communication). Nelson (1935b) reported that the hypophysectomized guinea pig showed the usual mammary response to oestrone, and Lyons (personal communication) has obtained similar results. However, Selye, Collip, and Thomson (1935) were unable to obtain mammary development in hypophysectomized rats and similar results have been obtained by Reece, Hill, Gomez, and Turner (personal communication). Nelson (unpublished) has observed no consistently significant development of the glands in hypophysectomized rats injected with oestrone only, but in some instances the combination of oestrone and progesterone induced a considerable degree of proliferation of alveoli as well as ducts. Houssay (1935b) has reported proliferation of the mammary glands in one hypophysectomized male dog which had received treatment with oestrone.

This problem obviously needs further investigation. It may be shown eventually that the pituitary does exert a direct influence on mammary development. However, the lack of development of the mammary glands in some animals is not proof that the hypophysis exerts a direct effect on the glands. The removal of the hypophysis affects so many physiological functions that it would not be surprising to find a developmental process involving many possible factors more or less influenced. The recent findings of Gaunt (p. 511) relating the adrenal cortex to the function of the mammary glands may be important in this regard.

THE EFFECT OF THE LACTOGENIC HORMONE IN HYPOPHYSECTOMIZED ANIMALS. In establishing the function of an endocrine gland in any

specific reaction, it has been an established procedure to remove the gland, observe the deficiency effects, and attempt to repair that deficiency by the administration of an extract of the gland. In a preceding section (p. 507) the invariable termination of lactation by hypophysectomy has been noted. It is reasonable to suppose that the administration of the lactogenic hormone would maintain or reëstablish lactation in the hypophysectomized animal. However, evidence is rapidly accumulating which indicates that the administration of potent lactogenic extracts does not serve as a complete replacement for the animals' own pituitaries. Lyons, Chaikoff, and Reichert (1933) observed the initiation of lactation in a dog injected with a pituitary extract of good lactogenic potency, but with little or no gonadotropic or growth hormone. McPhail (1935a, b) was able not only to initiate lactation in the ferret and the cat, but was able to reinstate it in one ferret with a crude pituitary extract. Riddle, Bates, and Dykshorn (1933) obtained lactation in two rats with their lactogenic extracts. However, Selye, Collip, and Thomson (1934) have been unable to maintain lactation in rats, even with daily implants of fresh rat pituitaries. Nelson (unpublished) has been unsuccessful in maintaining lactation in the rat with either crude extracts of the pituitary or purified lactogenic preparations. In the guinea pig a few instances of the reinstatement of lactation have been observed, but only in animals receiving a crude extract which contained demonstrable amounts of most of the known pituitary hormones. Purified lactogenic preparations were ineffective. Turner, Hill, Gomez, and Reece (personal communication) have been unable to maintain or revive lactation in cats, rats, guinea pigs, or goats. Houssay (1935a, b) was able to induce lactation in 4 of 5 hypophysectomized bitches, and in one male that had received preliminary treatment with oestrone. He was also able to revive lactation in 2 of 3 parturient bitches after their normal lactation had been interrupted by hypophysectomy.

At this time it is impossible to interpret the lack of agreement in the findings noted above. Variability in the animals and in the pituitary extracts employed may be found accountable for the differences. It is worthy of note that removal of the adrenal glands, which are dependent in part at least upon the anterior pituitary for their maintenance, will interrupt lactation.

OTHER ENDOCRINE RELATIONS IN LACTATION. *The adrenals.* Carr (1931) reported that adrenalectomy interrupted lactation in the rat. The injection of an extract of the adrenal cortex maintained the life

of the operated rats, but would not support lactation. Gaunt (1933) found that rats adrenalectomized during pregnancy might deliver normal litters, but only rarely would lactate sufficiently to rear them. This, despite the fact that the mothers might be showing no marked symptoms of adrenal insufficiency. Swingle and Pfiffner (1932) found that adrenalectomized bitches maintained on cortical hormone would conceive, deliver, and lactate in an apparently normal fashion. Brownell, Lockwood, and Hartman (1933) reported that their cortical extract, cortin, would support life, but not lactation in rats. If, however, they added to their regular extracts, or failed to remove from them, a fraction of fatty substances, lactation was supported in the majority of instances. They looked upon their results as an indication for a separate cortical hormone, cortilactin. In a short note Britton and Kline (1935) reported that even partial adrenalectomy in rats and cats may interfere with lactation, and that in the rat their cortical extracts would support milk secretion. Houssay (1935b) stated that removal of the thyroid gland, the ovaries, and the adrenals did not interfere with lactation in the dog.

The recent unpublished observations of Dr. Robert Gaunt, which he has kindly placed at my disposal, would appear to clarify some aspects of the problem. With his permission I am summarizing these findings. Rats, adrenalectomized late in pregnancy, will deliver and show a transient lactation, but only rarely will give enough milk to raise their litters. Maintenance doses of the Swingle-Pfiffner cortical extract likewise failed to support normal lactation, but huge doses were effective. Salt, given in the diet and drinking water, was ineffective, but in combination with small quantities of cortical extract served to maintain lactation. In repeating the experiments of Selye, Collip and Thomson (p. 505) the adrenals were also removed. A transient lactation sometimes occurred after oöphorectomy, but unless salt solution was given it appeared to be less than in the controls. Gaunt's interpretation of these findings is a logical one. In adrenalectomized animals a primary disturbance appears to be the loss of fluid and salt from the blood serum. Consequently the large fluid shifts and secretion necessary in lactation would quite likely be seriously impaired unless the deficiency is relieved by administration of the cortical hormone or by considerable amounts of salt solution. In other words, a dehydrated animal would not be expected to lactate.

In the light of these findings it is possible that the observed failures of pituitary extracts, particularly the refined preparations which do not

repair cortical damage, to maintain or initiate lactation in hypophysectomized animals may be due to an adrenal insufficiency. Different species and probably different animals of a given species undoubtedly suffer varying degrees of cortical damage following hypophysectomy. Further experiments in which cortical hormone, large amounts of salt solution, or both are given simultaneously with the lactogenic hormone would be of value. However, in this regard, it must be noted that although hypophysectomy does damage the adrenal cortex there is little evidence to indicate that a hypophysectomized animal suffers from the fluid and electrolyte disturbances which occur after adrenalectomy.

The posterior pituitary. It has been known for many years that the injection of extracts of the posterior pituitary would in some manner affect the secretion of milk (Ott and Scott, 1910). Many early workers, e.g., Simpson and Hill (1914), believed that the extracts acted as a true galactogogue and stimulated the secretion of milk, and Fauvet (1932) has recently claimed that the posterior lobe secretes the lactogenic hormone. However, the reports of Hammond (1913), Schafer (1914), and Turner and Slaughter (1930) would seem to have shown that the posterior lobe extracts have no actual action as a galactogogue. Instead, it is believed that their action is on the discharging mechanism and affects formed milk only. For a review of the earlier literature the reader is referred to the paper by Turner and Slaughter. The report of Smith (1932) that female rats whose posterior lobes had been removed, leaving most of the anterior lobe intact, would continue normal reproductive activity, conceive, deliver, and lactate normally appears to have established the non-essentiality of the posterior lobe in lactation. Houssay (1935a) has made similar observations on the dog.

The pancreas and thyroid. A relation of these glands to lactation is vague, but not unlikely. However, only a brief consideration of the subject will be possible at this time. Chaikoff and Lyons (1933) reported that only one of six depancreatized bitches, maintained on insulin, lactated when a potent pituitary extract was injected. However, Nelson, Himwich, and Fazikas (1936) were able to demonstrate that the reaction of depancreatized bitches to the lactogenic hormone depends upon the developmental condition of the mammary glands. Thus, animals with a pseudo-pregnant type of mammary development invariably lactated, while consistently negative results were obtained in animals with regressed or undeveloped glands.

Weichert, Boyd, and Cohen (1934) have noted that hyperthyroidism in the female rat induced a condition of pseudo-pregnancy and proliferation of the mammary glands. Weichert and Boyd (1934) previously had reported that the induction of a hyperthyroid condition in pregnant rats resulted in a more rapid development of the mammary glands and an earlier appearance of secretory products in the alveoli. The interpretation placed upon these findings by the authors is based on an increased activity of the hypophysis due to the less effective action of oestrone in the hyperthyroid animal. McQueen-Williams (1935a, b) has noted recently an exhibition of maternal behavior on the part of thyroidectomized male rats. Furthermore, she has found the hypophyses of such males less effective than those from normal animals in stimulating the pigeon crop-gland. This was believed to indicate that the hypophyses of the maternalized males had been drained of much of their lactogenic hormone. It is significant that Riddle, Lahr, and Bates (1935) have identified the lactogenic hormone as the factor responsible for maternal behavior.

Kustner (1934) reported that the suppression of the thyroid by the administration of an antithyroidal substance, "Tyronorman," increased lactation in women who, judged by their previous histories, were not expected to lactate normally. On the other hand, Graham (1934a) observed that thyroidectomy in the cow caused a slight decline in milk yield and a more significant decline in milk fat. The addition of desiccated thyroid to the diet of thyroidectomized and normal cows during the period of declining milk yield caused a rise in milk and milk fats. No effect was observed during the periods of rising yield. Later, Graham (1934b) identified thyroxin as the constituent of the thyroid responsible for these effects.

NERVOUS INFLUENCES IN LACTATION. Although most of the evidence has indicated a lack of nervous influences in lactation (for a review of the literature see Basch, 1906; Selye, Collip, and Thomson, 1934) certain recent findings have introduced new considerations. Cannon, et al. (1929), reported that complete sympathectomy in the cat had no effect upon the occurrence of oestrus, conception, pregnancy, or lactation, but later (Cannon and Bright, 1931) they noted a delayed effect of sympathectomy upon lactation. In the rat, Bacq (1932) found that removal of both abdominal and one thoracic sympathetic chains usually had no effect upon any of the reproductive functions, but observed an occasional disturbance of lactation.

In a series of papers, Selye (1934), Selye, Collip, and Thomson (1934),

and Selye and McKeown (1934a, b) a group of significant experiments were reported. It was found in the rat and mouse that it was possible to induce complete proliferation of the mammary glands in non-pregnant animals through mechanical stimulation of the nipples by an actively suckling litter. Furthermore, such animals actually lactated and were able to raise the litters of other mothers.[2] As the oestrous cycles were stopped or greatly prolonged the general reaction was termed "suckling pseudo-pregnancy." It was also observed that the stimulus of suckling would maintain active lactation over considerable periods of time, even in cases where removal of milk was prevented by ligation of the galactophores. On this basis it was held that the act of suckling reflexly stimulates the production of the lactogenic hormone by the hypophysis and that the involution of the mammary glands after weaning is conditioned by the withdrawal of the nervous stimulus of suckling rather than by the accumulation of the products of secretion. This latter view had been generally accepted and evidence in its favor offered for the rabbit by Hammond and Marshall (1925) and for the guinea pig by Kuramitsu and Loeb (1921) and Nelson (1934a). Selye's studies indicate the necessity for further consideration of the effect of suckling as an actual stimulus for lactation. A really critical experiment would appear to be the use of sympathectomized animals. Lactation failure in such cases would establish the importance of the suckling stimulus in maintaining lactation. Bacq's rats were able to rear their young in most instances, but his experiments are not entirely critical, in the present regard, since the pectoral glands on one side probably retained nervous connections and their stimulation might have sufficed to provide for the production of the lactogenic hormone. However, it would be possible to remove or cover the nipples in the region not subjected to sympathectomy.

In summarizing our knowledge of the nervous factors involved in the activities of the mammary gland it can be said that glandular development and lactation almost certainly are controlled by hormonic influences only (centrifugal phase), but that the possible centripetal phase of the mechanism is less clearly devoid of nervous factors.

[2] It is of interest to note that the reports of lactation in virgins might be explained on the basis of these experiments. Krestin (1932) has reviewed a number of these reports in addition to recording two cases of spontaneous lactation associated with enlargement of the hypophysis. Rivoire (1935), in connection with a general discussion of the subject, has considered the frequent association of galactorrhea and hypertrophy of the pituitary.

THE LACTOGENIC HORMONE. *Extraction methods.* The extracts used in the early experiments of Stricker and Grueter, Corner, and Nelson and Pfiffner were simply alkaline or acid extracts of whole pituitary glands from oxen and sheep, and contained a number of the pituitary hormones. However, Corner (1930) indicated that a separate hormone might be involved in the lactogenic response. Riddle, Bates, and Dykshorn (1932, 1933) introduced a step in purification by the use of isoelectric precipitation methods. They demonstrated that either acid (pH 1.5 to 2.5) or alkaline (pH 8.5 to 11.0) extraction[3] could be used successfully, but that the isoelectric point (point of minimum solubility) of the active substance varied accordingly with the method employed. The name "prolactin" was proposed for the active substance in their extracts.

Gardner and Turner (1933) also employed isoelectric precipitation after alkaline extraction in preparing their lactogenic hormone, which they termed "galactin." Lyons and Catchpole (1933a) employed acid-acetone extraction of acetone dried anterior lobes and precipitated the active material at a pH of 2.0. In a later paper (Lyons and Page, 1935) the term "mammotropin" was given to their lactogenic hormone preparations. Using intradermal injections in pigeons they were able to detect a response in the crop-gland with a 0.001 mgm. dose.

A method that involves the repeated extraction of the material, obtained by isoelectric precipitation, with glacial acetic acid, followed by the addition of ether to reprecipitate the active substance, is being employed in a number of laboratories. McShan and Turner (1935) have recently published their use of this procedure. They note that a ten-fold concentration of the lactogenic hormone is obtained and that 3 mgm. will induce a significant increase in the weight of the pigeon crop-gland.

Bates and Riddle (1935) have recently described a method of purification that depends upon the initial extraction of pituitaries with alkaline alcohol, separation of the lactogenic hormone by isolectric manipulations in aqueous solution, and final purification in 70 per cent alcohol. Preparations were obtained which gave detectable crop-gland responses with 0.02 mgm. doses.

Pituitaries from cattle, sheep, and hogs have been the principal source of the lactogenic hormone used in experimental studies. Riddle, Bates,

[3] The loss of activity encountered by Anselmino and Hoffmann (1934) with 0.1 N sodium hydroxide solutions (about pH 13.0) seems to indicate the danger of highly alkaline extraction.

and Dykshorn (1933) rated them in the above order for their relative potency. Although whole pituitaries are usually extracted, Gardner and Turner (1933) reported that in one experiment they removed the posterior lobes from a batch of beef pituitaries before extracting them. The response obtained with this material was identical with that observed when whole glands were extracted.

Bates, Riddle and Lahr (1935) assayed pituitaries from seven classes of cattle for lactogenic hormone. Per gram of moist tissue, 26–34 pigeon units were found in glands from veal calves, adult steers, adult bulls, and non-pregnant cows; 38 units in glands from cows in early pregnancy, 44 units in glands from cows in late pregnancy, and 78 units in glands from fetuses. Lyons and Page (1935), on the basis of tests in pigeons, report that as much hormone is excreted daily by a lactating woman as is extractable from a bovine anterior lobe. Lyons (personal communication) states that detectable amounts of lactogenic hormone are present in as little as 100 cc. of normal male urine. In cases of teratoma testis and granulosa cell tumor of the ovary large quantities of the hormone were recovered from the urine.

Chemical and physical properties. Relatively little is known as yet concerning the nature of the lactogenic hormone. That the hormone is a protein was indicated by its destruction following tryptic digestion (Bates, Riddle and Lahr, 1934). McShan and Turner (1935) state that their best preparations gave positive biuret tests. These studies, of course, do not entirely exclude the possibility of the adsorption of the active substance on inert proteins. The hormone is not destroyed by heating for one hour in boiling water at pH 7.5 to 8.5, but is destroyed at a higher or much lower pH, or in the presence of salts (Riddle, Bates, and Dykshorn, 1933). Anselmino and Hoffmann (1934) state that the hormone is very heat labile since the activity of their preparations was destroyed by heating at 60 degrees for 15 minutes. However, with their method of extraction it seems probable that the final solutions were acid (about pH 3.0). The active substance will not dialyze through a collodion membrane (Gardner and Turner, 1933). The finding (Anselmino and Hoffmann, 1934) that the active principle is non-ultrafiltrable through 8 per cent acetic acid collodion probably indicates a larger molecule than in the case of the other anterior lobe factors. On the other hand, this may be evidence in favor of adsorption of the active substance on inert protein. It is stable in the dried condition or in solution at low temperatures for a considerable period of time. However, as noted by Riddle, Bates, and Dykshorn, an

optimum pH and absence of salt are in favor of stability of the hormone in solution.

Assay methods. Riddle and Braucher (1931) showed that anterior pituitary extracts would cause the crop-glands of pigeons and doves to undergo proliferation and to produce "crop milk.' On this basis, Riddle, Bates, and Dykshorn (1932, 1933), after noting that the crop-gland response in birds and lactation in mammals were induced by the same extracts, suggested the use of the crop-gland response as the basis of a method for the quantitative assay of the lactogenic hormone. Injections of the material under test are made once daily for four days and the crop-glands are removed and weighed on the fifth day. Employing the data obtained on a given extract as a standard, the logarithm of the dosage is plotted against the crop-gland weights, which are corrected for body weight, and the unit defined as that amount of prolactin which is equivalent to 1 mgm. of the standard preparation in producing an increase in weight of the crop-glands. This method has been used generally as an assay procedure. It is attractive because of its quantitative nature and because the assay of an unknown preparation can be read from a previously constructed standard curve. However, the weight response of the crop-gland has been found so variable, particularly in the pigeons usually available, that this method of assay has led to considerable difficulty in attempts to purify and standardize the hormone. Realizing this deficiency, attention has been paid to the use of a minimum proliferation of the crop-gland as the criterion of assay. Lyons and Catchpole (1933a) were the first to suggest such a procedure and McShan and Turner (unpublished) have recently outlined a method of assay with a given degree of proliferation as its basis. Following the method outlined by Coward and Burn (1927) for biological assay, they were able to show that from the percentages of birds exhibiting a minimum proliferation of the crop-gland with increasing amounts of hormone, a characteristic sigmoid curve might be constructed. On this basis the unit is defined as that total amount which, when injected once daily for four days into each of 20 common pigeons weighing 300 $\pm$ 40 grams, will cause a minimum, but definite proliferation of the crop-glands in 50 $\pm$ 11 per cent of the birds.

Gardner and Turner (1933) used the pseudo-pregnant rabbit as a standard test animal. Such animals have a uniform type of mammary development, and when injected with lactogenic hormone will secrete milk abundantly. In the original test, animals were injected once daily for seven days and the glands examined and rated, according to

the extent of lactation within the gland, on the following day. A rabbit unit is the minimum amount which will induce a plus 3 or 4 response. In the reviewer's experience satisfactory assays can be conducted on a five day injection basis. This method is, on the whole, less variable than the crop-gland procedure, but depends upon the observer's judgment in rating the glands. Unpublished observations on the ratio between the pigeon and rabbit units have given figures varying from 15–1 to 100–1 with the average about 40–1. Such figures for different extracts might be taken as evidence for the existence of two separate hormones, but it is probable that they simply reflect the variability of the two tests.

Donahue (1934) examined the reactions of rabbits, in various stages of sexual development, to lactogenic hormone extracts and concluded that the intact multiparous female was the most suitable for assay purposes.

Lyons and Catchpole (1933a) suggested the use of adult virgin guinea pigs, which had been spayed during oestrus, for assay purposes. The test was judged positive if milk could be expressed by manipulation of the nipples. However, since Nelson (1932b, 1934b) has shown that adult female guinea pigs will lactate spontaneously a few days after removal of the ovaries it would seem that such animals are untrustworthy test subjects. It was suggested (Nelson, 1934b) that the intact adult female guinea pig might be used for assay purposes, but that a better subject was found in guinea pigs which had been hysterectomized during pregnancy. Such animals rank with the pseudo-pregnant rabbit in having mammary glands which are prepared for lactation.

Relation to other anterior lobe hormones. The crude extracts used in the pioneer studies on the lactogenic effect of pituitary preparations also produced a number of other effects attributable to the anterior lobe. However, there is now reasonable evidence for believing that the substance which induces lactation in mammals and proliferation of the crop-gland in pigeons may be a separate hormone. Riddle, Bates, and Dykshorn (1933) offered evidence indicating that the lactogenic hormone is distinct from the growth promoting and gonad-stimulating principles. Gardner and Turner (1933), Lyons and Catchpole (1933a), and Nelson (1934b) have noted that the lactogenic potency of pituitary extracts apparently had no relation to their content of gonad-stimulating hormone. It should be noted, however, that on the basis of the present evidence only the follicle-stimulating hormone can be said to be distinct from the lactogenic factor. No evidence

thus far reported has definitely shown that luteinizing and lactogenic factors actually exist as separate hormones. Riddle (1933) and Riddle, Bates, and Lahr (1935) state that their prolactin preparations are free of thyreotropic hormone, and Nelson (unpublished data) has seen no evidence of thyroid stimulation in guinea pigs injected with moderate amounts of similar extracts. While there is very little direct evidence on the adrenotropic effect of lactogenic hormone preparations, Riddle, Lahr, Bates, and Moran (1934) have noted that their prolactin caused an increase in the weight of the rat adrenal. Nelson, Turner, and Overholser (1935) have shown that the administration of lactogenic hormone to rabbits and monkeys was without significant effect upon the blood sugar level. Lyons (personal communication) states that he has obtained preparations which are free of the other recognized principles of the anterior pituitary.

Specific responses attributed to the lactogenic hormone. In addition to the effects of this hormone on the mammary and crop glands, which have been considered in detail, several other interesting phenomena, resulting from the injections of lactogenic hormone, have been observed. Riddle, Bates, and Lahr (1935) and Bates, Lahr, and Riddle (1935) were able to identify the lactogenic hormone as the substance concerned in the induction of broody behavior—the incubation instinct—in fowl. Associated with, indeed preceding, the expression of broodiness, was a cessation in the production of eggs and a regression of the ovary. A logical extension of this discovery was the observation (Riddle, Lahr, and Bates, 1935; Riddle, 1935) that the administration of their prolactin preparations induced maternal behavior in virgin rats. Wiesner and Sheard (1933) had suggested that some pituitary principle probably was concerned in the excitation of maternal behavior, but the involvement of the lactogenic hormone was considered unlikely.[4]

Riddle and Bates (1933) reported that their prolactin extracts would induce a rapid involution of the adult dove testis, an effect presumably associated with the period of incubation and proliferation of the crop-glands. Riddle, Lahr, Bates, and Moran (1934) found that prolactin injections in adult male rats had no significant effects on testes or sex-accessories.

Riddle, Smith, Bates, Moran, and Lahr (1936) made the interesting

[4] Recently Leblond and Nelson (Anat. Rec. **64**: 29, 1936, suppl.) have shown that hypophysectomy has no significant effect upon the development or maintenance of maternal behavior in mice and rats.

observation that prolactin would raise the basal metabolic rate in normal, hypophysectomized, and thyroidectomized pigeons. Thyreotropic hormone produced this effect only in the presence of the thyroid. It was further noted that the effects commonly attributed to a specific growth hormone could be obtained in pigeons by the combined action of the lactogenic and thyreotropic fractions.

Clinical use of the lactogenic hormone. Few reports on clinical trials of the lactogenic hormone have appeared, although a number of attempts have been made to increase milk secretion in parturient women. Kurzrock, Bates, Riddle, and Miller (1934) reported the treatment of 37 women, delivered of normal children, with prolactin. Only one or two injections were given and the total dosage in no instance exceeded 400 pigeon units. Although the majority of women showed a slight increase in lactation the results are, as Werner (1935) has pointed out, of doubtful significance since the injections, in most instances, were made at the time lactation usually shows a spontaneous increase.

Werner (1935) attempted to induce lactation in 8 women who had been oöphorectomized some time previously. Six of the subjects had received oestrone or oestrone and progesterone prior to treatment with the lactogenic hormone. In no instance was lactation observed, although enlargement of the breasts was noted in all subjects. The total dosages of lactogenic hormone ranged from 200 to 2600 pigeon units over periods of 1 to 14 days. Although these reports offer little encouragement for clinical success in the use of the lactogenic hormone, it seems probable that the use of more potent preparations will lead to satisfactory results.

The report of Chausselat (1935) on the oral administration of sera from lactating cows and mares in cases of deficient lactation in women is encouraging, but rather surprising in view of the finding (Bates, Riddle, and Lahr, 1934) that the lactogenic hormone is destroyed by tryptic digestion. In 24 of 30 instances a definite increase in milk secretion was observed. Sera from pregnant cows or mares were without effect. It is significant that the subsequent use of sera from lactating animals in these cases induced an increase in lactation. In addition to his experimental work, Chausselat reviewed the clinical literature on hypo- and hyper-function of the mammary glands.

The possible use of the lactogenic hormone in the treatment of mammary lesions as suggested by Geschickter, Lewis, and Hartman (1934) is interesting, but as yet has little factual basis.

SUMMARY

It may be of value to summarize briefly, and in a very general fashion, certain facts which at this time appear to be fairly well established concerning the hormonic control of the mammary glands. 1. The development of the mammary glands proceeds under the influence of the ovarian hormones. The different degrees of development present in males, non-pregnant females, and pregnant females are due to different degrees of stimulation by oestrone and progesterone. 2. The initiation and maintenance of lactation depends upon the presence of the anterior hypophysis. Lactation apparently is controlled by an anterior lobe hormone specifically related to milk secretion and possibly to the associated behavior of the mother. 3. The ovarian hormones, which are present in unusual quantities during pregnancy, are responsible for mammary proliferation, but inhibit the secretion and action of the lactogenic hormone. With the decline in level of the ovarian hormones at parturition the inhibiting influences are removed, the lactogenic hormone is secreted, and lactation occurs. 4. Although the development of the glands and the initiation of milk secretion appear to be under purely hormonic control, it seems probable that the maintenance of lactation is influenced by a nervous mechanism in the reflex stimulation of the hypophysis.

A great deal of work remains to be done in clarifying much that is as yet less clearly established, or purely conjectural. Some of these outstanding questions deserve mention. 1. The possibility remains that the anterior pituitary does participate in the development of the mammary glands; this, of course, in addition to its action through the ovaries. The nature of the possible pituitary contribution is vague, but probably the lactogenic hormone is not involved. 2. Mammary development during pregnancy may be divided into proliferative and secretory phases. The proliferative phase is reasonably well understood, but the mechanism controlling the secretory phase is less clearly delineated. It is possible that the secretory phase occurs as a result of some change in the relations between the ovarian hormones, or through some effect of the lactogenic hormone acting in conjunction with the ovarian hormones. 3. Further exploration of the action of oestrone on the inhibition of lactation, and the relation between the activities of oestrone and the lactogenic hormone at the time of parturition, are desirable. 4. Additional data are needed on the relation of nervous influences, and of the thyroid and adrenal glands to lactation.

5. Complete elucidation of the exact relation of the hypophysis to the development and function of the mammary glands depends upon the more thorough understanding of the different hormones of the anterior lobe and upon the judicious employment of these in suitable experiments.

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